LIPID ACCUMULATION AND UTILIZATION DURING MICROCYCLE GROWTH OF *ASPERGILLUS NIGER*

BY

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A THESIS SUBMITTED TO CNAA IN PARTIAL FULFILMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

RESEARCH CONDUCTED AT SHEFFIELD CITY POLYTECHNIC IN COLLABORATION WITH THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH, MORETON, MERSEYSIDE

OCTOBER 1986

Advanced studies, courses and conferences undertaken and attended in connection with the programme of research in partial fulfilment of the requirements for the degree of Ph.D.

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2. 6 - 7th September, 1981

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3. March - June, 1984

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2. November 1980 - June 1984

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Conferences attended

1. 22nd April, 1981

   Varian Associates seminar and demonstration on "The role of high performance liquid chromatography in the analysis of proteins and peptides". Manchester University.

2. 13 - 16th April, 1982

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3. 14th December, 1982

   Biodeterioration Society conference at Sheffield City Polytechnic.

4. 11 - 15th April, 1983

   British Mycological Society Conference on "The Ecology and Physiology of the Fungal Mycelium" at the University of Bath where I presented a poster communication on 'Lipid accumulation and utilisation during microcycle growth of Aspergillus niger'.

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Student teaching was undertaken on the following courses in the Biological Sciences Department, Sheffield City Polytechnic, and consisted of the following:-

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3. October - November 1983

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   3 hours per week.

A number of students were instructed in their special projects for HND and B.Sc. courses. These projects involved some practical application of microbiology and/or biochemistry.

ACKNOWLEDGMENTS

My sincere thanks are extended to my supervisors Dr. Ian Spencer and Dr. Barry Davis for their inspiration, guidance, cheerful optimism and patience throughout this investigation and thesis preparation. I also wish to thank them for their critical appraisal of the thesis in its final form.

I am greatly indebted to the technical staff of the Biological Sciences Department, Sheffield City Polytechic for their assistance and advice on experimental work and Mageurite Lyons for her help in typing part of the thesis. Thanks are also due to Sheffield Local Education Authority for funding the Research Assistantship.

My sincere thanks to Andy Atkinson for assisting in the preparation of the thesis. I wish to thank Ian McKay for this quality graph reproductions, Wendy Carr for her excellent typing and Tim Farrow for his first-class diagrams.

Finally, I am extremely grateful to my family and friends, particularly Liam Kennedy for their constant support and encouragement.

ABSTRACT

LIPID ACCUMULATION AND UTILIZATION DURING MICROCYCLE GROWTH OF ASPERGILLUS NIGER

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Aspergillus niger was grown in fermenter culture under conditions promoting microcycle growth. Following a period of spherical growth at elevated temperatures for 24h, conidiophores developed from the swollen giant cells when the temperature was decreased. Each stage in this microcycle conidiation was photographed and dry weight was determined. Aberrant growth forms sometimes occurred and these are described with the measures taken to attempt to minimize such problems.

The lipid content and composition was investigated throughout the microcycle by the use of column and thin layer chromatography and by gas-liquid chromatography. The major classes of neutral lipid were triacylglycerols, fatty acids, sterols and sterol esters. Changes in composition during the microcycle are discussed in relation to metabolic requirements for the different developmental stages and a function for triacylglycerol as an energy reserve for conidiation is suggested. The fatty acid composition was also determined throughout the cycle and changes related to growth temperature.

The accumulation and utilization of triacylglycerol was indicative of changes in activity of lipolytic enzymes. However little lipase activity was detected, although enzymes which hydrolysed water-soluble esters were more readily assayed.

In order to assess the relative utilization of each of the carbon substrates glucose, L-glutamate and L-alanine, they were provided in a radiolabelled form and the fate of the label followed at intervals throughout the cycle. The majority of the material was used in the production of insoluble cellular material, with smaller amounts incorporated into lipids, water-soluble materials or released as carbon dioxide. Very little label from L-glutamate was detected as lipid. Glutamate was principally used during the later, conidiation, stage of the microcycle. The results are discussed in relation to the different physiological stages of microcycle conidiation and to the observed changes in lipid content and composition.

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ABBREVIATIONS USED IN THE TEXT

^{14}C	Carbon fourteen isotope
cm	centimetre
Co.	company
CO_2	carbon dioxide
DEGS	di-ethyl glycol succinate
dpm	disintegrations per minute
E.C.	enzyme code
Ed.	editor
Eng.	English
g	gramme
h	hour
H_2SO_4	suphuric acid
$KHCO_3$	potassium hydrogen carbonate
l	litre
M	molar
Me	methyl
mg	milligram
min	minute
ml	millitre
N_2	nitrogen
nm	nanometre
plc	public limited company
pp.	pages
rev.	revolution
sec	second
tca	trichloroacetic acid

tlc	thin layer chromatography
w/v	weight to volume ratio
µg	microgram
µl	microlitre
%	percentage
^{o}C	degrees Centrigrade

The various members of the genus Aspergilli feature prominently in many aspects of research into fungal biochemistry. They are also important in industry, including the medical and pharmaceutical industries and have applications in biotechnology. Some members of the genus have roles in soil chemistry and in aspects of food spoilage and biodeterioration. Species of Aspergilli are frequently isolated from soil, vegetation, air and certain substances undergoing biodeterioration (Raper and Fennell, 1965; Hudson, 1969).

Many of the Aspergilli are agents of biodeterioration and are particularly common in soil where they degrade organic material. Aspergillus niger has been isolated from wood surfaces (Scheffer, 1973) and its extensive cellulolytic activity is known to cause strength loss to cellulose strips (King & Eggins, 1973). The Aspergilli are of major economic significance since they are major deteriogens of cellulose and thus of wood and vegetable fibres. Although the growth of these spoilage organisms must be retarded to keep the losses in the food, cosmetic and pharmaceutical industries to a minimum, their use as biodeteriogens may be exploited to solve waste disposal problems and in the recycling of raw materials from refuse.

A. niger is particularly important in industry as a producer of organic acids. The main organic acid produced by A. niger is citric acid and many fermentation techniques are employed to carry out the process. Traditionally, industry has used the surface culture technique although greater emphasis is now being placed on the use of submerged culture and also of continuous culture (Berry, 1977). A. niger is also used in the production of industrial enzymes such as amyloglucosidase and pectinase.

Amyloglucosidase attacks amylose and amylopectin and its main use in industry is to hydrolyse starch completely to glucose. Pectinases are used extensively in the wine and juice industries and contain a mixture of several pectic enzymes which together split the pectin molecule into units of galacturonic acid.

Various species of Aspergilli have been used for thousands of years in the Orient to convert indigestible and unappetising ingredients into palatable and nutritious foods. The main species used in modern fermentations is Aspergillus oryzae which produces a fungal amylase. The conversion of otherwise bland vegetable protein to flavoured, nutritious products by fungal fermentation is likely to contribute to industrial food processing and production in the near future to satisfy the demand for foods from a rapidly increasing world population.

A range of fermentation techniques are used in industry for the production of enzymes, antibiotics and other economically important substances from fungi and they include solid-state, submerged and continuous culture. Such techniques usually result in a heterogeneous fungal population with different cells at varying stages of development. These techniques were also used to grow fungi for biochemical studies on fungal growth cycles and the results obtained thus represent an average over a range of cell types and ages. However, the discovery of the growth cycle known as microcycle conidiation in A. niger (Anderson & Smith, 1971, a;b) has allowed the study of a relatively homogeneous culture of fungal cells undergoing differentiation. During this cycle, the A. niger spores grow spherically at an elevated temperature for twenty four hours to produce large cells termed giant cells.

On lowering the incubation temperature, the giant cells develop conidiophores which produce conidia as illustrated in Fig. 3.2. Various aspects of biochemistry have been studied during microcycle conidiation in A. niger. Protein and RNA biosynthesis have been studied by Duncan et al. (1978), nucleic acid levels and nuclear numbers by Yahya (1979) and Smith et al. (1981 a;b). The morphological aspects of microcycle conidiation in A. niger have been investigated by Anderson & Smith (1971, a;b) and the causes of autolysis in the giant cells studied by Kuboye et al. (1976). The chemical structure of the cell wall during microcycle conidiation in A. niger has been examined by Deans (1978) with particular attention to α-glucan, β-glucan, chitin, galactomannan, protein and lipid content.

With the exception of the investigation into total lipid levels in the cell wall by Deans (1978) there have been no reports of the lipid content of A. niger cells undergoing microcycle conidiation. The primary concern of the present thesis was to investigate the lipid content of A. niger cells undergoing microcycle conidiation. The results obtained are presented in four chapters, each complete with its own introduction and literature review, experimental data and discussion. All experimental procedures are detailed in the Materials and Methods section, Chapter 2.

The morphological aspects of microcycle conidiation in A. niger are examined in Chapter 3. This chapter also deals with variations in microcycle growth. The lipid content of the spores, giant cells and conidiating structures during the various stages of microcycle conidiation is investigated in Chapter 4.

The total cellular lipid content was estimated and the extracted lipids separated into neutral and polar lipids by column chromatography and the amounts present quantified. The neutral lipids were further separated into their constituent classes by thin layer chromatography and quantified by densitometry. The fatty acid composition of the total lipid and of the triacylglycerol fraction was determined by gas chromatography. The cells were tested for the presence of esterase, which have been previously reported in A. niger mycelial growth (Lloyd et al., 1971; 1972), and a lipase analysis was also undertaken. The results of these enzyme analyses are detailed in Chapter 5.

The fate of radiolabelled carbon supplied as the incubation medium substrates, ^{14}C glucose, ^{14}C glutamic acid and ^{14}C alanine was investigated in Chapter 6. The carbon radioisotope was traced into the cell, particularly into the cellular lipid fraction and the cells' carbon dioxide emissions were monitored. The radiolabelled carbon was also traced through into the individual neutral lipid classes separated by thin layer chromatography. The combined conclusions of the individual chapters are detailed in Chapter 7.

2.1 Organism

The organism used throughout the study was Aspergillus niger. The main species used was Aspergillus niger van Tiegham, University of Strathclyde strain provided by Dr Barry Davis, Sheffield City Polytechnic, Pond Street, Sheffield, but supplementary experiments were undertaken using Aspergillus niger IMI numbers 41873, 50565 and 96215.

2.2 Media and Culture Conditions - A. niger conidia

2.2.1 Stock Cultures

A. niger was mainly grown on slopes of potato glucose agar but occasionally on potato carrot agar slopes. The composition of both media is detailed in Table 2.1. The potato glucose agar was prepared using the stock obtained from 800g of potatoes as follows. The potatoes were washed thoroughly, cut into small pieces and boiled in 2 l of distilled water for 1h. The solid material was separated from the stock by filtration through four layers of cheesecloth. The stock was then added to 2 l of distilled water containing 80g of glucose and 80g of agar. The final solution was made up to 4 l with distilled water and placed in a steamer to dissolve the agar (Table 2.1).

Potato Glucose Agar (PGA)

Potatoes	800g
Glucose	80g
Agar	80g
Water	4 l

Potato Carrot Agar

Peeled Potatoes	80g
Scraped Carrots	80g
Agar	60g
Water	4 l

Table 2.1 Media composition for stock slope cultures of A. niger

Medium constituents

Sodium glutamate	5.00g
Ammonium sulphate	2.00g
Potassium dihydrogen phosphate	1.00g
Magnesium sulphate	0.25g
L-alanine	0.20g
Calcium chloride	0.047g

Salt solution

Ferrous sulphate	6.30g
Manganese chloride	3.50g
Zinc sulphate	1.10g
Copper sulphate	0.24g

The salt solution consisted of the above ingredients in 1 l of distilled water.

Table 2.2 Growth medium for A. niger giant cell cultivation.

The culture medium contained the above medium constituents in 1 l of distilled water together with 1ml of the salt solution described below. In shake tube cultivation, 10g of glucose was added directly to the medium but in fermenter cultivation the glucose was autoclaved separately to avoid caramelization. The pH was checked and adjusted to pH 4.5 where necessary.

The potato carrot agar was prepared by macerating 80g of peeled potatoes and 80g of scraped carrots and placing in 4 l water. The resultant mixture was boiled for 0.5h and strained through cheesecloth, and 60g of agar was added to the stock.

In both cases, the hot medium was dispensed in 10ml aliquots into Universal bottles which were autoclaved for 15min at 121^{o}C and then sloped. The slopes were aseptically inoculated with spores from existing stock cultures and incubated at 30^{o}C for 4 - 6 days and then stored at 4^{o}C until required for use.

The purity of the stock cultures was checked at regular intervals.

2.2.2 Spore Production

Large quantities of spores for inoculation purposes were obtained by growing A. niger on bread. Preservative-free white bread was cut into cubes of approximately 1cm^{3} and 30g was placed in each 250ml Erlenmeyer flask. The flasks were placed in an oven for 30min at 60^{o}C to dry the bread, fitted with cotton wool plugs and then autoclaved at 121^{o}C for 15min.

A. niger conidia were obtained aseptically from the stock culture slopes using sterile distilled water. The flasks were inoculated with a fine spray of these conidia introduced via a sterile syringe, and then incubated at 30^{o}C for up to 8 days.

The conidia were harvested from the bread cubes by the addition of 100ml sterile distilled water per flask and, after shaking vigorously, were filtered through several layers of sterile muslin to remove all traces of bread and hyphae.

The spores were washed five times in sterile distilled water by centrifugation at 15,000rpm in a Beckman J2-21 centrifuge fitted with a JA-20 fixed angle rotor head for 10min at 10°C. They were then re-suspended in sterile distilled water, shaken on a Griffin flask shaker for 10min to disperse clumps and counted in a haemocytometer chamber to determine the spore concentration. They were then stored at 4°C until required for use.

2.3 Culture Medium - A. niger giant cells

The medium used was as described by Anderson and Smith (1971;a,b) and is detailed in Table 2.2.

2.3.1 Shake tube culture (boiling tube cultivation)

The culture medium, including the glucose, was dispensed in 7.0ml aliquots into boiling tubes (15 x 2.5cm) which were plugged with cotton wool and autoclaved for 15min at 121°C. On cooling, each tube was aseptically inoculated with 7×10^6 spores and the cotton wool plugs replaced by sterile rubber bungs. The tubes were incubated in a Gallenkamp orbital shaker operating at 250rev/min for 24h. The rubber bungs were then replaced by sterile cotton wool plugs to allow aeration and the temperature was reduced to 30°C for a further 24h to complete the microcycle.

2.3.2 Fermenter Cultivation

A greater biomass of homogeneous culture was produced using a Gallenkamp Modular Fermenter. The fermenter system consisted of a 1 l culture vessel with cover plate and access ports connected to stirrer, pH and temperature control modules and a water-cooled outlet condenser. The vessel was fitted with a millipore filtration system consisting of a pre-filter and a membrane filter. This was connected via a rotating float flow meter to an air/carbon dioxide mixing unit or gas cylinder, which ensured that the gas supply entering the culture vessel was sterile. The gas mixture used was 5% carbon dioxide in air flowing into a ring sparger at a rate of 200ml/min, manually set on the flow meter. The air/carbon dioxide ratio was set using a gas mixing unit which had inflows from a carbon dioxide cylinder and from an air compressor. A back-up supply of a 5% carbon dioxide in air cylinder was coupled to the system and automatically took over when the carbon dioxide cylinder emptied.

Dispersal of the gas mixture and agitation of the culture medium was initially provided by a magnetic drive stirrer which was subsequently replaced by a direct shaft stirrer module. The module consisted of a geared drive motor enclosed in a plastic case secured above the fermenter. The drive motor was connected by a flexible rod to a shaft-stirrer gland assembly attached to the central port of the vessel's cover plate. The stirrer turbine, located at the end of the shaft was a disc with four blades on both its surfaces. The agitation speed was set at 400rev/min.

The assembled fermenter vessel containing a working volume of 800ml of medium, but lacking glucose, was autoclaved at 121^{o}C for 40min. The glucose was autoclaved separately in 40ml of medium at 121^{o}C for 10min, so preventing caramelization. The medium, with the glucose aseptically added, was allowed to equilibrate at the initial incubation temperature of 41^{o}C before a spore inoculum of 1 x 10^{6} conidia per ml was introduced via one of the free ports.

The incubation temperature over the initial 11h of giant cell production was 41^{o}C, increasing to 44^{o}C for the subsequent 13h. A reduction in temperature to 30^{o}C at 24h ensured the completion of the microcycle. Initially, temperature control was provided by a Compenstat solid - state thermostat, thermistor sensing probe, immersion heater and cooling coil. A mercury in glass thermometer was also fixed in one of the ports and read at regular intervals to confirm the set temperature. However, it was found that this system did not always monitor and compensate for small changes in temperature to which the A. niger giant cells were sensitive. Consequently, the temperature module system supplied with the fermenter was replaced by a Tecam TE-7 Tempette proportional temperature controller. This allowed temperature equilibration of the vessel contents internally and externally. The culture vessel was placed in a water bath, the contents of which were equilibrated and continuously circulated through a coil immersed in the fermenter medium.

2.4 Harvesting of Cell Samples

Samples of the required volumes were aseptically removed at times throughout the microcycle, or, for analyses requiring a greater biomass, the entire vessel contents were taken. The samples taken from both the shake tubes and the fermenter were centrifuged at 20000rpm for 10min in a Beckman J2-21 centrifuge fitted with either JA-20 or JA-10 fixed angle rotor heads, depending on the sample size. They were then washed twice with sterile distilled water using the same centrifugation conditions. The cells were resuspended in trichloroacetic acid in order to inhibit any enzyme activity. However, in enzyme assays the cells were re-suspended in distilled water for esterase estimation and in buffer for lipase detection.

2.5 Morphological Observations

Nomarski interference microscopy was used to produce photographs of the various stages in microcycle conidiation. An Olympus Vanox microscope with an attached Olympus C-35AD camera were employed in accordance with the manufacturer's instructions.

2.6 Cell Wall Breakdown

2.6.1 X-Press

Some degree of cell wall breakdown was achieved using an AB Biox X-press (Nacka, Sweden).

A cell suspension in trichloroacetic acid was poured into one of the X-press chambers which had been pre-cooled in liquid N_2. The sample froze immediately and was forced through a small hole connecting the two chambers using a 12 tonne pressure exerted by a hydraulic press. This caused a change in the crystal structure of ice which fragmented material in the sample accompanied by a loud cracking noise. The frozen suspension was passed from one chamber to the other until no further cracking sounds were heard. It is apparent from Table 2.3 that a cell wall breakdown procedure is required in order to release the lipid present. The giant cells tested were grown for the same length of time, one lot was tested untreated, the other after the X-press cell breakdown procedure. Lipid was extracted and analysed as explained in Sections 2.7 and 2.8.

2.6.2 MSK Braun Rotary Homogeniser

More efficient cell wall breakdown was achieved using a MSK Braun rotary homogeniser. Here the samples were placed with an equal volume of glass beads of diameter 1.00-1.05mm in enclosed Duran glass bottles ranging in size from 12-70ml. The bottles, which have a ground joint stopper with a special retainer were mounted in rubber buffers inside a steel tube whose axis carried out a circular motion at a speed of 4000rpm for 20sec. The cooling temperature was controlled by the inflow of liquid CO_2 and a significant increase in cell wall breakdown was achieved by the bombardment of the cells with the ballatini.

Cell wall breakdown was observed under a phase contrast microscope and was found to be approximately 75% with the Braun treatment, although this does not include cells with hairline fractures which would not be visible under these magnifications.

2.7 Lipid Extraction

Lipid was separated from non-lipid material by a method based on that of Bligh & Dyer (1959). Chloroform/methanol (1:2, v/v) was added to the homogenised sample in a separating funnel and allowed to stand for 24h at $4^{o}C$. Water and chloroform were then added to give a two phase system with overall composition of chloroform - methanol - water, 10:10:9 (by volume). The stoppered funnel was then shaken vigorously for 1min. The two phases usually separated on standing although centrifugation was sometimes necessary.

The lower, chloroform layer containing the extracted lipid was removed and dried down in a waterbath at $45\text{-}50^{o}C$ under a gentle stream of N_2 to prevent oxidation of the lipids. Alternatively the chloroform was removed from larger samples using a rotary evaporator (Edmund Büehler).

The extracted lipid was then dissolved in chloroform and any particulate matter present was removed by filtration through glass micro-fibre filter paper.

2.8 Total Lipid Analysis

The total amount of lipid present in a given sample was measured using the sulphuric acid oxidation method of Marsh & Weinstein (1966).

A nitrogen dried lipid sample in a test tube was heated with 1.0ml concentrated sulphuric acid for 15min at 200^{o}C in a metal heating block. On cooling, 1.5ml of distilled water was added to the samples, left for 10min and then thoroughly mixed. The absorbance at 375nm was determined on a Pye Unicam SP1800 spectrophotometer. A linear standard curve was obtained using known amounts of olive oil as the standard.

2.9 Neutral and Polar Lipid Analysis

2.9.1 Column Chromatography

The polar and neutral lipid fractions were separated using column chromatography. A Pasteur pipette containing glass wool at the tapered end and filled with 0.25g silicic acid (Bio-rad silicic acid for column chromatography -325 grade) served as a column. Chloroform was stirred into the silicic acid which was then allowed to drain and pack. The extracted lipid dissolved in 0.5ml chloroform was applied to the drained gel followed by 2 washes with 0.5ml chloroform. The neutral lipids were eluted with 3ml chloroform, the polar lipids with 3ml methanol. The amount of the lipids in both the effluents was then determined using the sulphuric acid charring method, having taken to dryness with N_2 as previously described.

The recovery efficiency of the columns was tested using known amounts of standard lipids.

2.9.2 Thin-Layer Chromatography

The plates used were obtained pre-coated with silica gel $60F_{254}$ (Merk) and were washed before use by running in chloroform/methanol (4:1, v/v) for at least an hour and then air dried. The polar and neutral lipids separated by column chromatography were taken to dryness with N_2 and re-dissolved in 50 µl chloroform/methanol (4:1, v/v). These were then applied to the t.l.c. plates using a capillary tube, followed by 3 x 50 µl washes of chloroform/methanol to effect quantitative transfer.

The polar lipids were separated using a chloroform:methanol : water (25:10:1, by volume) solvent system, and the neutral lipids in a freshly prepared solution of light petroleum spirit:diethyl ether: glacial acetic acid (80:30:3, by volume). The lipids were located by spraying with 50% H_2SO_4 using an aerosol attachment and placed in an oven at 100-110^{o}C for 1h. The resultant charred spots were quantified using a Joyce Loebl Chromoscan and a photographic record obtained using a Zenith 35mm camera. Lipid standards of known composition were obtained from Sigma. Fig. 2.1 shows a typical separation obtained and also its chromoscan trace.

2.10 Fatty Acid Analysis

Fatty acid composition was determined using gas chromatography of the methyl esters of the acids. Preparation of methyl fatty acid esters by transesterification was by a modification of the method of Christie (1972). Dichloromethane (0.20ml) was added to a dried sample of lipid, followed by 0.2ml of a sodium methoxide solution (2M in methanol).

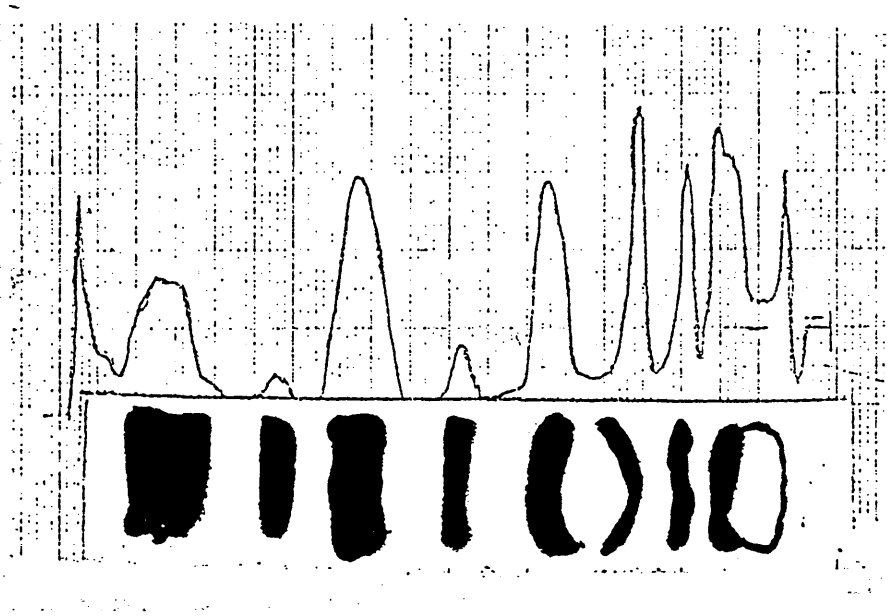


Fig. 2.1 The separation, by thin layer chromatography of the lipid classes present in the A. niger cells undergoing microcycle conidiation. The trace was obtained using a Joyce Loebl Chromoscan and a diagrammatic representation of the charred areas of various lipid classes is illustrated.

The samples were heated at 50°C for 15min, 0.01ml glacial acetic acid and 0.5ml water were added and the mixtures extracted with 3 x 1.0ml diethyl ether. The ether extracts were washed with 1.0ml sodium bicarbonate solution (2%, w/v) and then dried over anhydrous sodium sulphate. The samples were taken to dryness under N_2 at 30°C, and the residues were taken up in dry petroleum ether. Separation and quantitation was by isothermal gas chromatography initially using a Perkin-Elmer gas chromatograph fitted with a flame ionization detector and a 10% DEGS (di-ethyl glycol succinate) column.

Later work utilised a Varian gas chromatograph attached to a computer integrator and fitted with a 10% DEGS column. The FID detector temperature was set at 220°C with an oven temperature of 195°C, a nitrogen flow of 40ml/min and a range of 10.

Figs 2.2 and 2.3 show a typical trace obtained on the chromatograph using standard solutions and a giant cell methylated fatty acid sample at 36h.

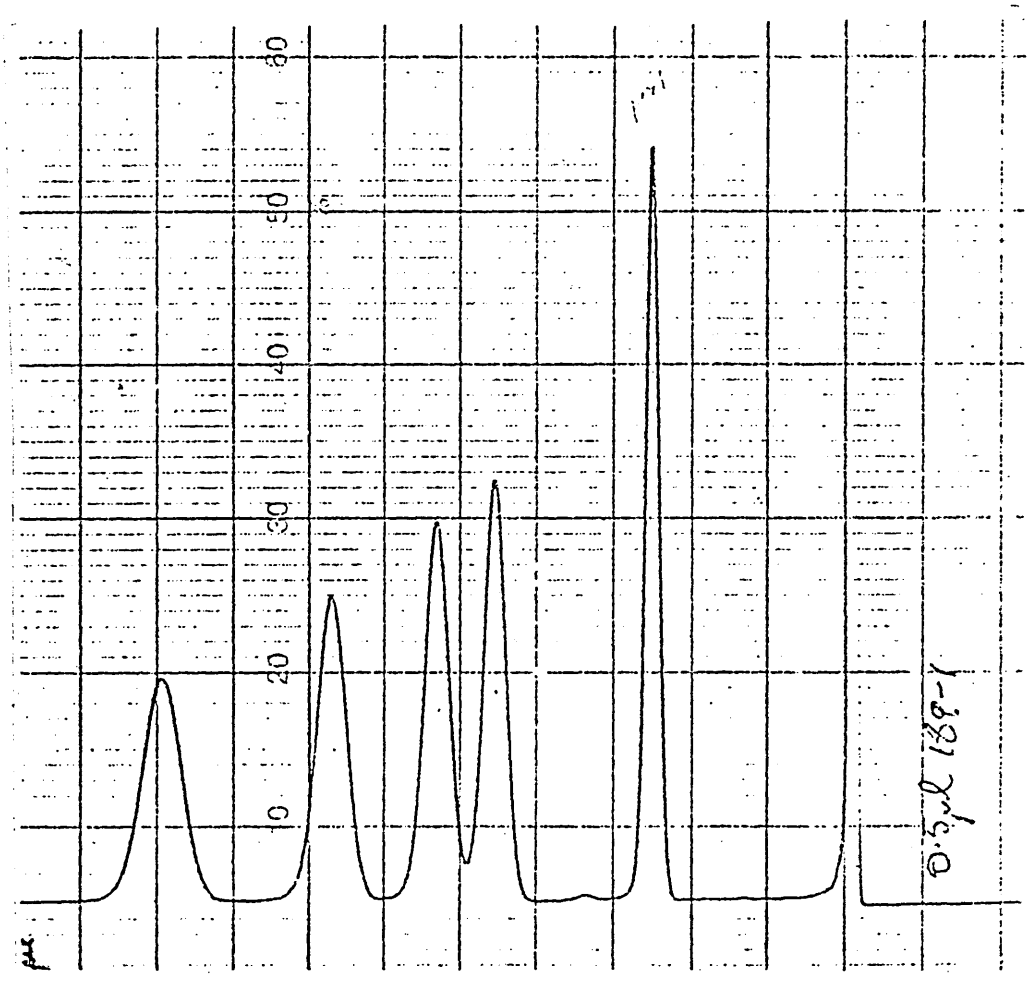


Fig. 2.2 The trace obtained when a standard fatty acid solution was separated by gas chromatography. The peaks represent, from right to left, palmitic, stearic, oleic, linoleic and linolenic acids.

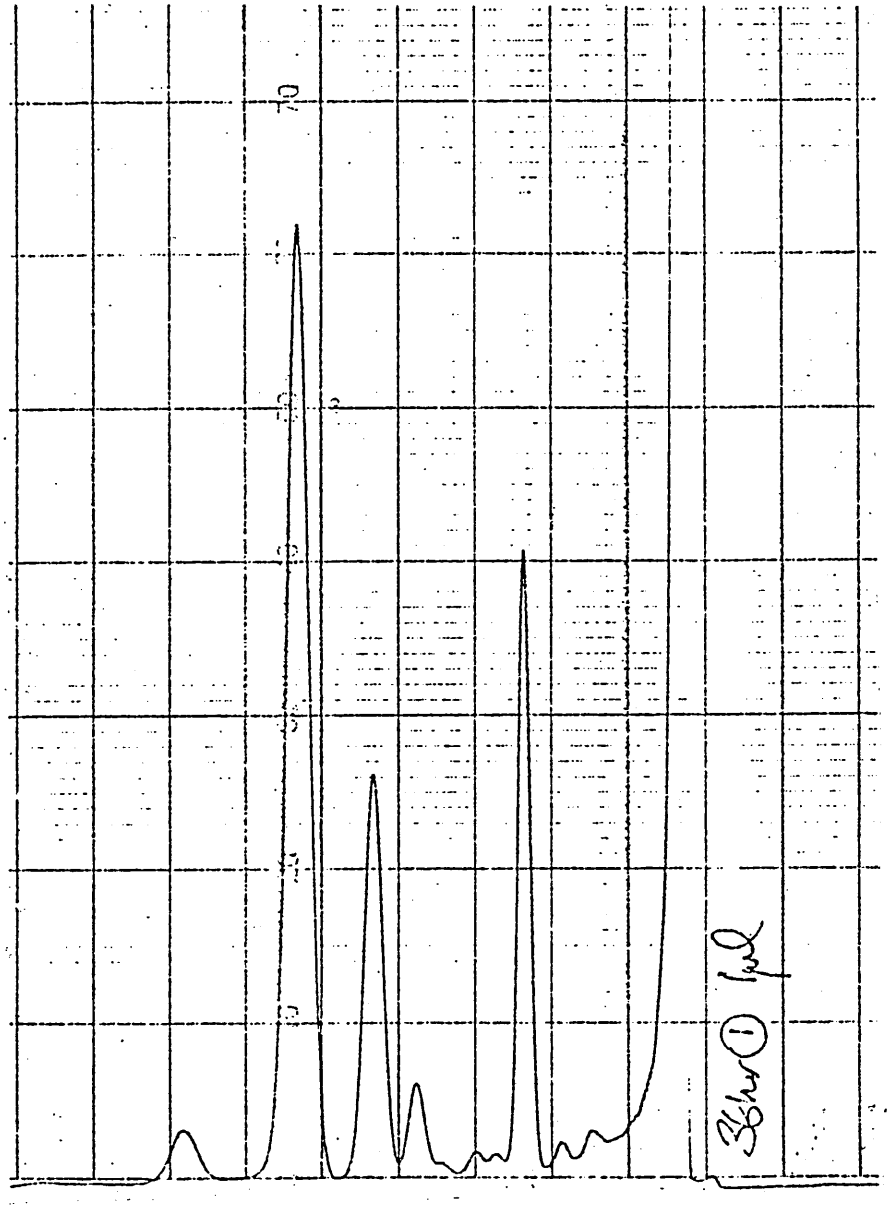


Fig. 2.3 The trace obtained on separating, by gas chromatography, the fatty acids extracted from A. niger cells, 36h into microcycle conidiation.

Cell wall breakdown treatment	Lipid content per 1 x 10^6 Cells ($\pm$ standard error for 3 replicates)
none	3.77 $\pm$ 1.60
X-press	6.21 $\pm$ 0.86

Table 2.3 Comparison of total lipid extracted from intact giant cells and from giant cells treated by the X-press cell wall breakdown process. Cultivation conditions were identical in each case, lipid extraction was as explained in Section 2.7 and lipid estimation by the sulphuric acid charring method of Section 2.8.

2.11 Protein Determination

The Braun homogeniser treatment was also used prior to protein assays of the cell suspensions. Total protein content was determined by the method of Lowry, Rosebrough, Farr and Randall (1951), using Folin-Ciocalteau's reagent. The absorbance was read at 680nm using a Pye Unicam SP1800 spectrophotometer, and a standard curve plotted using known concentrations of bovine serum albumin.

2.12 Glucose Determination

The amount of glucose present in the medium at various stages over the 48h cycle was measured by the GOD Perid method using glucose oxidase reagent obtained from Boehringer Mannheim. A standard curve was produced using known amounts of glucose read in a spectrophotometer at 436nm.

2.13 Determination of ninhydrin reactive material

The amino acid content (and other reactive amino compounds) of the fermenter medium was measured by the ninhydrin method using a modification of the procedure of Moore and Stein (1948). A suitably diluted 0.1ml aliquot of sample was mixed with 1.0ml of ninhydrin reagent. Each test tube was covered with a glass ball to prevent solvent evaporation. After heating in a boiling waterbath for 20min, 5.0ml of diluent solution was added with mixing. After 15min the absorbance was determined at 570nm in a Pye Unicam SP1800 spectrophotometer. A standard curve was prepared using known concentrations of L-alanine.

2.14 Dry Weight Measurement

Whatman No 1 filters were dried overnight in an oven set at 50-60^{o}C. These were weighed prior to filtering 40ml from the fermenter culture vessel at various stages throughout the microcycle. The filters containing the cellular material were dried to constant weight in an oven set at 50-60^{o}C, and the weight of the cells determined by difference.

2.15 Radioisotope Tracer Experiments

2.15.1 Collection and Preparation of Samples for Radioisotope Counting

The fate of various carbon sources supplied in the media was traced using ^{14}C labelled isotopes. The fermenter was set up as previously described in Section 2.3.2, but prior to spore inoculation the required amount of ^{14}C labelled substrate was added. A 10ml sample of medium was taken at this stage for scintillation counting.

Great care was taken to ensure that no radioisotope contamination occurred during the course of these experiments. Procedures were carried out over plastic trays and rubber gloves were used throughout.

The radioisotopes supplied as substrates in the A. niger growth medium were D-(U-^{14}C) glucose, L-(U-^{14}C) glutamic acid and L-(U-^{14}C) alanine. The radioactive concentrations were 200 μCi/ml or 7.40 MBq/ml for glucose, 50 μCi/ml or 1.85 MBq/ml for alanine and glutamic acid.

2.15.2 Collection of Radiolabelled Carbon Dioxide

It was essential that the fermenter culture vessel be completely airtight in the radiotracer experiments, and that any radiolabelled carbon dioxide produced should be absorbed. The only outlet for the gases from the culture vessel was through the water-cooled outlet condenser which was connected to two air tight cylinders in succession. Each cylinder contained 15ml ethanol and 31ml ethanolamine which absorbed all the carbon dioxide emitted from the culture vessel. The first ethanol/ethanolamine cylinder was replaced each 12h in order to ensure that all the carbon dioxide was absorbed.

2.15.3 Preparation of Radiolabelled Carbon Dioxide Samples for Scintillation Counting

Any of the ethanol/ethanolamine mixture lost due to evaporation during the course of microcycle fermentation was replaced in the original proportions. Then 0.5ml aliquots were removed from each cylinder and placed in scintillation counting vials. Ethanol (1ml) was added to each vial followed by 10ml of cocktail 'T' and the mixture thoroughly agitated. Cocktail 'T', Scintran contains the following constituents per litre.

Toluene	666ml
'Triton x-100'	332ml
2, 5-Diphenyloxazole (PPO)	5g
1, 4-Di-2 (5 phenyloxazolyl) - benzene (POPOP)	0.15g

2.15.4 Collection and Preparation of Medium and Whole Cell Samples

The giant cells were sampled, harvested and homogenised as previously described in Sections 2.4 and 2.6. In order to evaluate the amount of radioactivity in the giant cells as a whole, it was first necessary to dissolve the cellular material. Samples of the giant cells (1ml of culture) were centrifuged in small plastic conical tubes at 2000rpm, the supernatant collected, and the cells transferred into scintillation counting vials. The cell material was dissolved by the addition of 1.5ml NCS tissue solubilizer (a solution of a quaternary ammonium base in toluene), the scintillation vial covered with cling film (Nesco-film), the cap screwed down tightly and the tubes heated at 50^{o}C overnight. On cooling, 10ml cocktail 'T' was added to each vial.

The supernatant resulting from the centrifugation of the harvested giant cells was collected and 0.5ml aliquots of this growth medium were placed in scintillation counting vials, to which 5.0ml of cocktail 'T' was added.

2.15.5 Collection and Preparation of Lipid Samples

Lipid extraction from the harvested giant cells was as previously described in Sections 2.7 and 2.8, with samples from the aqueous layer of extraction being retained for scintillation counting. One tenth of the resulting lipid in chloroform solution, from an initial harvest of 60ml of culture, was dried down using a steady stream of nitrogen gas in a scintillation vial placed in a waterbath at 45-50^{o}C.

The lipid was re-dissolved in 1ml of ether, 5ml of cocktail 'O' was added and the vial shaken. Cocktail 'O' Scintran is a liquid scintillator solution in toluene containing 6g of 2, 5-Diphenyloxazole (PPO) and 0.2g of 1-4, Di-2-(5-Phenyloxazolyl)-benzene (POPOP) per litre.

The radioisotope content of the aqueous layer of lipid extraction was determined using 0.5ml aliquots to which 5.0ml of cocktail 'T' was added.

2.15.6 Scintillation Counting

The radioactive content of all the prepared samples was repeat counted using a Nuclear Enterprises 8312 scintillation counter. The counter was programmed to record the time taken for 10,000 radioactive decays, or should this time be greater than 600sec, the number of decays occurring during the latter time interval was noted. Counting efficiency was estimated by the channels ratio method and counts converted to disintegrations per minute (d.p.m.).

2.15.7 Radioisotopes traced to Lipid classes separated out by Thin Layer Chromatography

The air tight fermenter system was set up as previously described in Section 2.3.2 and the cells harvested at 12, 24, 36 and 48h. The lipid was extracted (see Section 2.7) from the harvested cells and also from a sample of spores. Separation of the lipid classes was attained using t.l.c. plates prepared as in Section 2.9.2.

Samples of the total lipid extraction were dried using nitrogen gas and re-dissolved in 50 µl chloroform/methanol (4:1, v/v). One sample and one standard lipid solution was spotted on each plate as previously described (Section 2.9.2) and the separation of the neutral lipid classes was attained using a chloroform/methanol/water solvent system. The polar lipids remained at the origin using this solvent system. The lipid bands were located using iodine as any permanent stain could interfere with the scintillation counting procedure.

The solvent system was allowed to evaporate from the t.l.c. plates for at least 10min. The plates were then placed in glass chromatography tanks containing 1g iodine, until all the lipid bands were coloured. The plates were exposed to the iodine for the shortest time possible and on removing, the outline of the bands was traced using a pencil. As iodine also interferes with the counting procedure, it was necessary to eliminate all traces of the yellow/brown bands by heating in an oven at $35^{o}C$ until all the stains disappeared.

The silica gel containing each individual lipid band was scraped from the t.l.c. plate and collected in separate scintillation counting vials. The radioactivity contained in each band was counted following the addition of 1.0ml diethyl ether and 5.0ml cocktail 'O' to each vial.

2.16 Enzyme Assays

2.16.1 Lipase Assay (E.C.3.1.1.3)

Lipase activity was estimated by a modification of the method of Nilsson-Ehle and Schotz (1976). Any lipase present in the harvested and prepared A. niger cultures was tested for the ability to attack radiolabelled (^{14}C) triolein to release radiolabelled free fatty acids, over a range of pH values.

(i) Harvesting and Preparation of A. niger Enzyme Sample

A series of fermenter systems were set up as previously described (Section 2.3), and the entire culture harvested at 12, 24, 36 and 48h. A spore sample was also taken. The cell samples were harvested as previously described in Section 4. The supernatant i.e. the growth medium, was poured off and stored on ice for analysis, and the precipitated cell samples separated into six lots and washed twice using distilled water followed by centrifugation as before. Each sample was re-suspended in 5ml of the appropriate buffer (pH 4, 5, 6, 7, 8 or 9) consisting of varying proportions of 0.2M anhydrous di-sodium hydrogen phosphate and 0.1M citric acid. The samples were then homogenised using a Braun MSK Rotary Homogeniser at 4000rpm for 20sec and placed immediately on ice.

(ii) Commercial Lipase Standard

A commercial enzyme (triacylglycerol lipase from Candida cylindracea) was used as a standard in the assay. The lipase was prepared by dissolving 0.100g of the enzyme in tris buffer. The tris buffer consisted of 2.4228g tris base dissolved in 150ml distilled water at 37°C. The pH was adjusted to pH 7.7 with dilute hydrochloric acid and made up to 200ml at 37°C. The pH was re-checked and the buffer stored on ice until required. A dilution series using the lipase solution and this buffer was prepared.

(iii) Substrate Preparation

The substrate for the assay consisted of 0.21ml glycerol tri (1-^{14}C) oleate stock solution (56.5mCi/mmol, 500 µl in 5ml chloroform) and 0.42ml of unlabelled triolein solution consisting of 92.65mg/ml. The mixture was placed in a small bottle (4 x 1cm), and dried under a gentle stream of nitrogen in a waterbath at 65°C.

A gum arabic solution was prepared by the addition of 2.0g of the powder to 15ml distilled water. The pH was adjusted to between 7 and 8 with a few drops of potassium hydroxide, the solution made up to 20ml, filtered and the pH re-checked. The dried substrate with 2.1ml of the gum arabic solution was sonicated in six, 10sec bursts using a MSE sonicator at an amplitude of 8 microns peak to peak. The sonicator probe was placed 6mm below the surface of the gum arabic/substrate solution in the small bottle which was immersed in cold water for cooling.

(iv) Fatty Acid Free Albumin

The fatty acids released by the lipase in the assay were bound using a 10mg/ml fatty acid free albumin solution. A range of bovine serum albumin solutions was prepared using the sodium hydrogen phosphate/citric acid buffer at the following pH values: 4, 5, 6, 7, 8, 9 and additionally using the tris buffer at pH 7.7.

(v) Free Fatty Acid Assay

The assay consisted of 50 µl albumin buffer, 50 (or 100) µl homogenised A. niger cells in the corresponding pH buffer to that of the albumin plus 100 µl substrate solution in gum arabic. A standard calibration curve (Fig 2.4) was produced using the commercial lipase in place of the homogenised A. niger cells. The assay constituents were mixed in glass conical centrifuge tubes which were kept on ice until each assay was prepared. Incubation was for 30min in a 37^{o}C waterbath, following which the tubes were placed on ice and the reaction stopped by the addition of 3.25ml of the extraction solvent (28.2ml methanol, 25.0ml chloroform and 20.0ml heptane).

Potassium hydrogen carbonate buffer, 0.1mol/l, was prepared by dissolving 1.00g anhydrous $KHCO_3$ in 70ml distilled water. The pH was adjusted to 10.5 using 1.0mol/l potassium hydroxide, the solution made up to 100ml, shaken and the pH re-checked. The free fatty acid extraction was completed by the addition of 1.05ml of the potassium hydrogen carbonate buffer to the assay tubes which were stoppered and whirlimixed for 15sec. Separation of the two extraction phases was achieved by centrifugation at 2000 rpm for 10min.

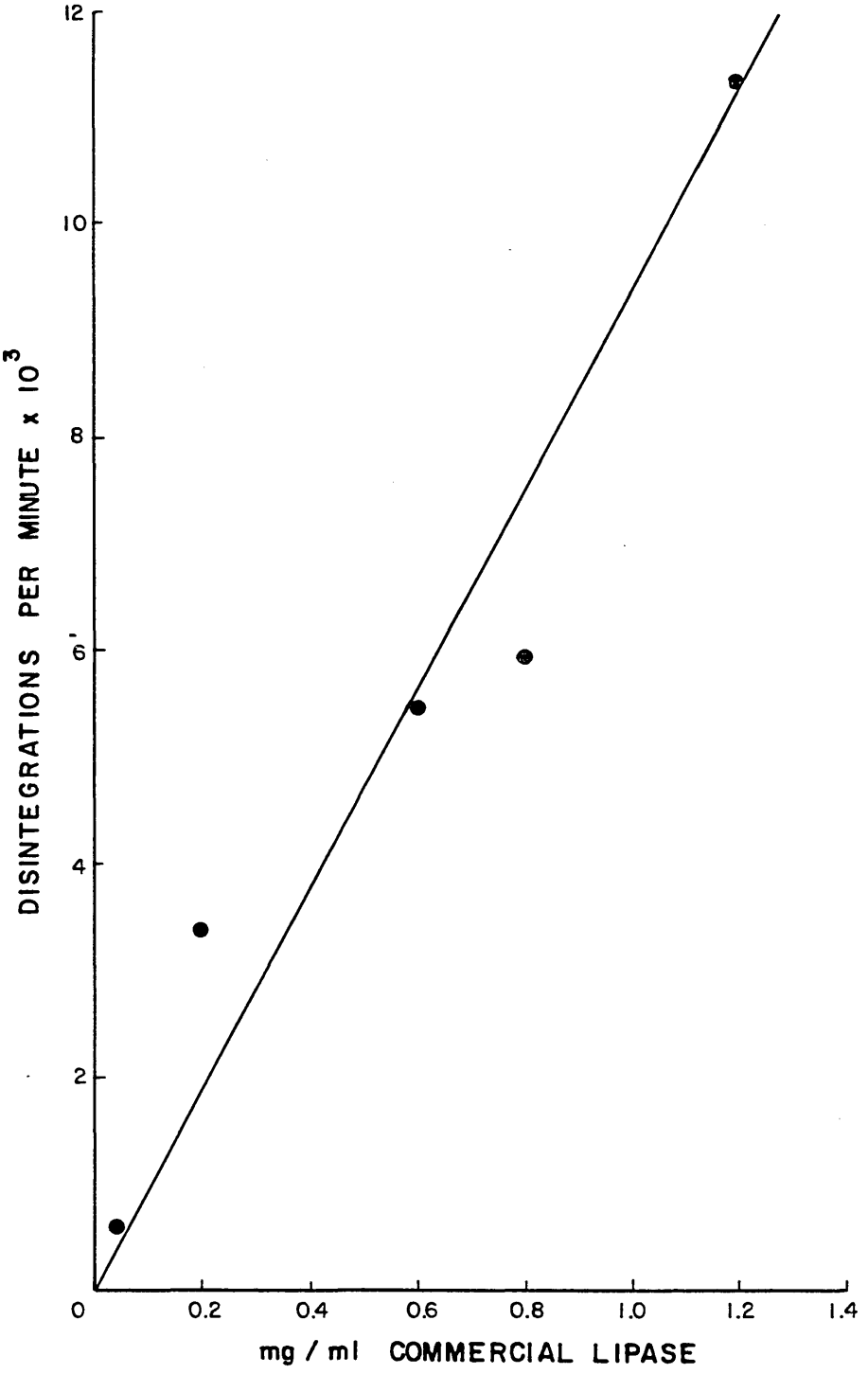


FIG. 2.4 CALIBRATION CURVE FOR THE ACTION OF COMMERCIAL LIPASE ON ^{14}C TRIOLEIN SUBSTRATE

Any free fatty acids released by the enzymes should be located in the uppermost phase of extraction, 1.5ml of which was pipetted out into a scintillation counting vial. The radioactive label incorporated into the fatty acids was recorded using a scintillation counter following the addition of 15ml cocktail 'T' and 1.5ml methanol to each vial. The results in dpm were converted to nmoles of free fatty acid for each assay.

2.16.2 Esterase Assay (E.C.3.1.1.1.)

Esterase activity was estimated in the A. niger cells by the method of Lloyd et al, 1971.

(i) Harvesting and Preparation of A. niger Enzyme Sample

The giant cells were harvested and homogenised as previously described in Sections 2.3 and 2.16.1. However some intact cells which had not been homogenised, were tested for esterase activity as were ungerminated spores and the cell-free growth medium from various stages in the microcycle.

(ii) Buffered-substrate Solution Preparation

A buffered-substrate solution was prepared, consisting of 10mg of 1-naphthyl acetate dissolved in 2ml acetone and 98ml 0.1M tris-maleate buffer, pH 6.4. The tris-maleate buffer consisted of 12.11g/l tris (hydroxymethyl) aminomethane and 11.61g/l maleic acid, the mixture adjusted to pH 6.4 by the addition of 0.1M sodium hydroxide.

(iii) Assay Procedure

The reaction mixture consisted of 6ml buffered-substrate solution and 1ml of giant cell or spore suspension in distilled water, or 1ml of the growth medium. The assay mixture was incubated at 30°C for 20min although some cells from the first 24h of the microcycle were assayed at their growth temperature of 44°C. A fast blue B salt (Gurr) solution, of 0.04g/l, was freshly prepared and 1ml of this added to the reaction mixture, followed by 1ml of 40% (w/v) trichloroacetic acid to facilitate extraction of the azo-dye from the protein-containing solutions.

(iv) Extraction of the Napthol Complex

Extraction of the complex formed by the naphthol released from the hydrolysed substrate with the dye was attained by the addition of 10ml ethyl acetate. The mixture was shaken, centrifuged at 2000 rpm for 10min in a bench centrifuge and the absorbance values of the upper layer of extraction, containing ethyl acetate and any red component of the purple azo-dye produced, were measured in a DMS 90 ultra-violet visible spectrophotometer (Varian) at 540nm.

(v) Calibration Curve

A linear calibration curve (Fig 2.5) was produced using 1-naphthol, and was used to convert the absorbance readings obtained in the assay to mg of 1-naphthol liberated. This was then converted to nmoles of product formed to allow comparison with the lipase results.

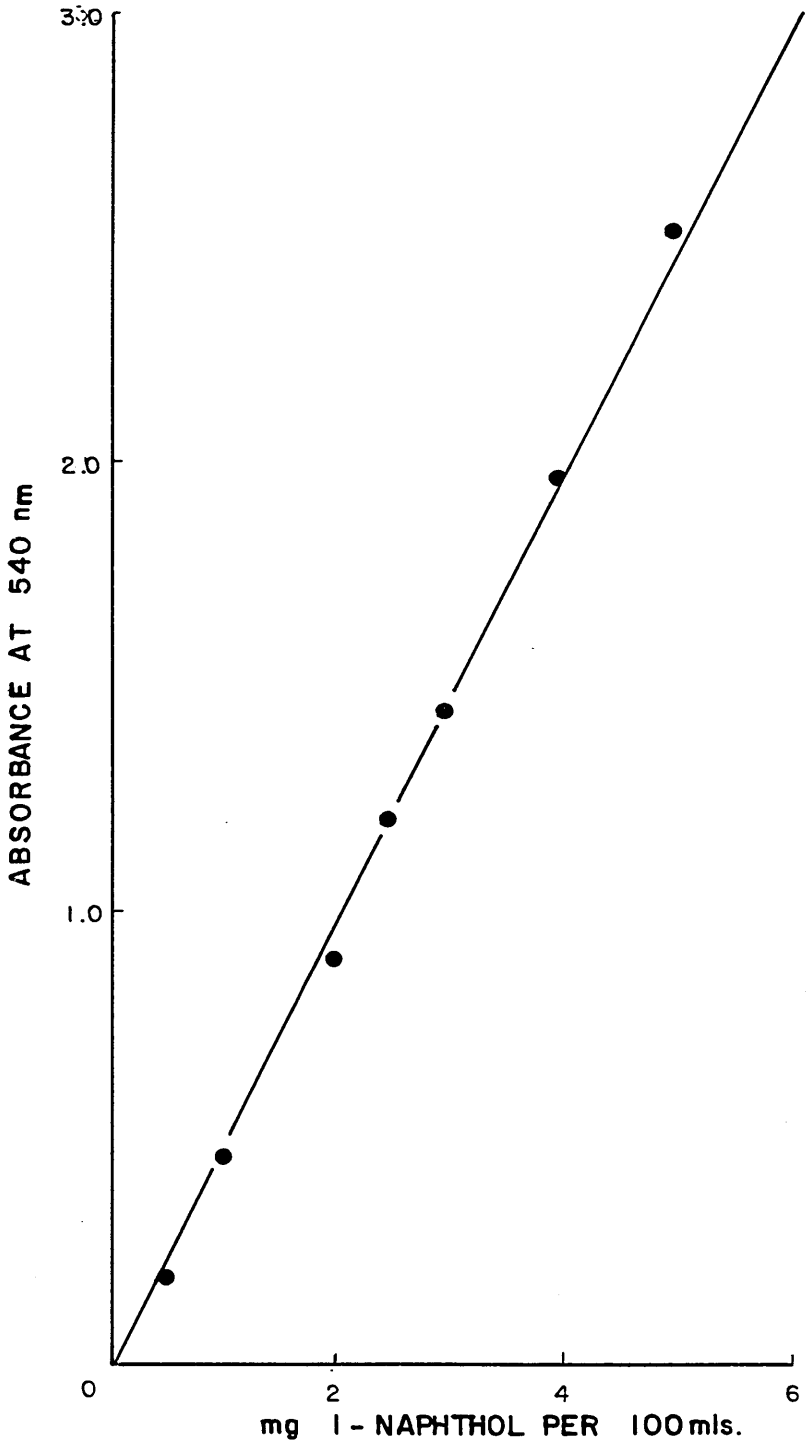


FIG. 2.5 CALIBRATION CURVE FOR ESTERASE ACTIVITY.

2.17 Source of Materials

2.17.1 Gases

Carbon dioxide (liquid and gaseous), 5% carbon dioxide in air and nitrogen gas filled cylinders were supplied by British Oxygen Company, Leeds, West Yorkshire, U.K.

2.17.2 Radioisotopes

^{14}C-labelled glucose, glutamic acid, alanine and triolein were supplied by Amersham International plc, Amersham, Buckinghamshire, England. NCS solubilizer for scintillation counting was also supplied by Amersham International plc.

2.17.3 Enzymes and Reagents

i) Glucose oxidase reagent was supplied by Boehringer Mannheim.

ii) Polar and neutral lipid standards used in t.l.c., and the esterified fatty acid standards used in gas chromatography were obtained from the Sigma Chemical Company, Poole, Dorset. The silicic acid used in column chromatography was obtained from Bio-Rad, 32nd and Griffin, Richmond, California.

The pre-coated, plastic, tlc plates prepared with silica 60 were manufactured by Merck and supplied via British Drug Houses.

iii) Other reagents were supplied by British Drug Houses, Poole, Dorset, U.K. and were of the highest standard commercially available.

3.1 INTRODUCTION

The biochemistry involved in the processes of fungal germination, filamentous growth, asexual and sexual development and metabolism has been studied in a number of organisms. The Aspergilli have featured in such studies (Smith & Anderson, 1973; Hastie, 1981) but rarely is one species of fungus of group of fungi suitable for the investigation of all aspects of growth and differentiation; as normal filamentous growth usually results in a heterogeneous population of cells representing a range of biochemical states.

The normal asexual development in Aspergillus niger is illustrated in Fig 3.1. The spore is involved at the beginning of the cycle where it functions to initiate vegetative growth and at the end where it represents a means of propagation, survival and dispersal. The alleviation of spore dormancy is followed by a period of swelling leading to germ tube emergence. Germ tube growth and branching results in the formation of a filamentous vegetative mycelium. The first stage in conidiophore formation is the transformation of a hyphal cell in the mycelium into a foot cell from which the conidiophore will emerge. The conidiophore is a single, erect, thick-walled stalk cell which develops perpendicular to the long axis of the mycelium and is usually unbranched and non-septate (Smith & Berry, 1974).

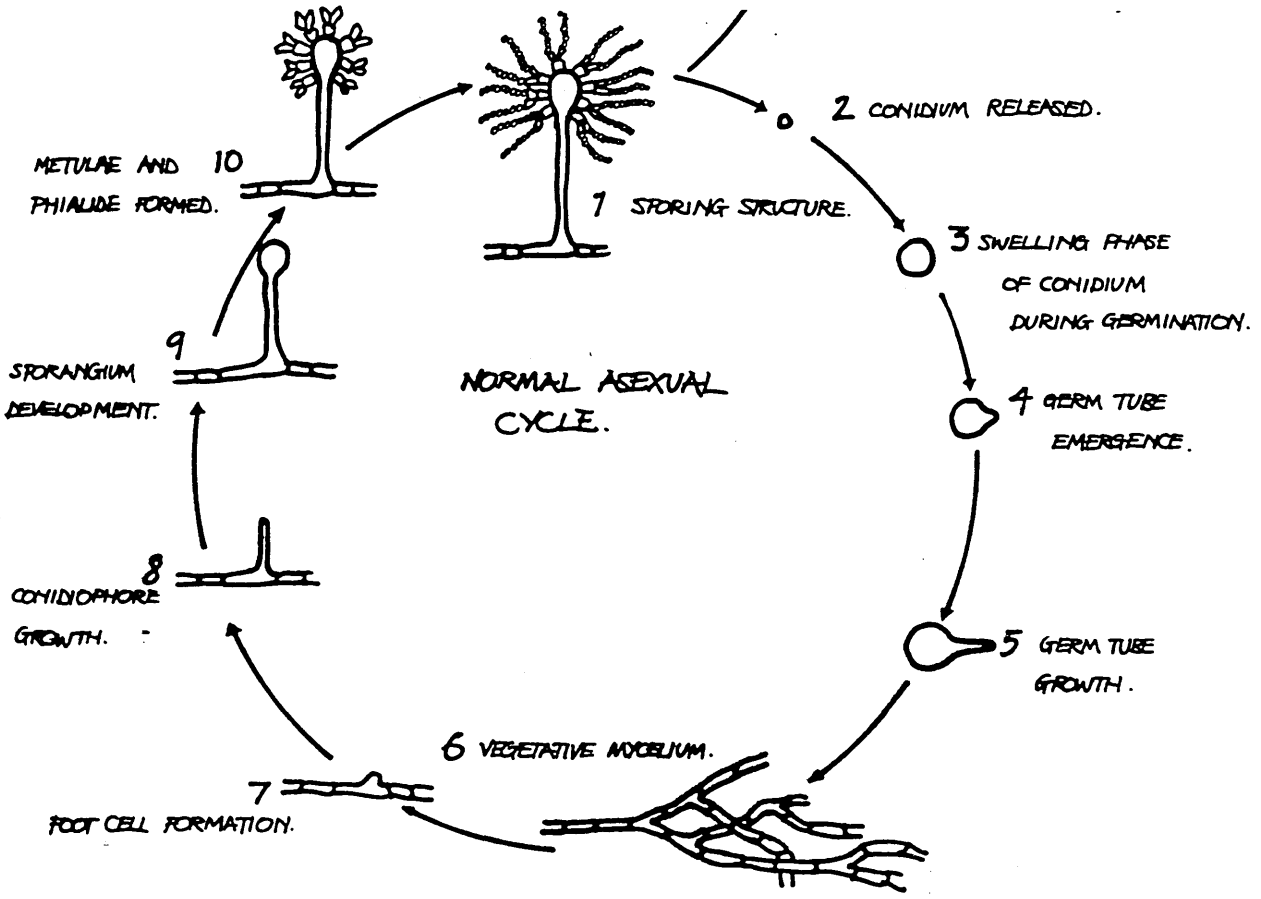


Fig. 3.1 Normal asexual development in A. niger.

When the conidiophore reaches its full length there is a gradual apical enlargement to form a vesicle, along with a thickening of the vesicle wall. Numerous primary sterigmata or metulae arise synchronously from the multinucleate vesicle (Tokunaga et al., 1973). A. niger produces 400-500 sterigmata which give rise to branched secondary sterigmata or phialides (Smith et al., 1977). The conidia develop in basipetal sequence from the phialide and are termed philaspores. The philaspores are formed by transverse septation and in aerial growth forms, long chains of conidia develop, held together by a common outer wall (Smith, 1978).

The traditional method of fungal cultivation is on the surface of a suitable medium. If the spore inoculum is dispersed evenly over the medium, a large hyphal mat of mycelium is formed giving rise to conidiophores of a uniform age. Conidiophore production may be suppressed if a layer of cellophane is placed over the mycelial mat; removal of the cellophane results in rapid and uniform conidiophore development. However, there is variation in the physiological state of the mycelial mat and whereas surface culture may be adequate for studies on environmental and genetic factors affecting conidiation, it is less suitable for biochemical and metabolic investigations into the stages of conidiophore production. Such limitations led to the development of submerged culture techniques, and conidiation in A. niger can now be achieved by shake flask culture as well as batch and continuous fermenter methods (Galbraith & Smith, 1969; Anderson & Smith, 1971; Ng, Smith & McIntosh, 1973a). Submerged culture techniques result in the growth of a more homogeneous mycelium particularly if growth is filamentous as opposed to pellet form.

In common with other fungi, Aspergilli grown in submerged culture will normally remain vegetative. However, foot cell development, the first step of conidiation, can be induced in A. niger in a medium where nitrogen is limiting but in the presence of a carbon source (Galbraith & Smith, 1969; Vezina & Singh, 1975). Conidiophore elongation will occur with the exhaustion of the exogenous nitrogen but with the continued presence of a carbon source. The addition of a nitrogen source together with citrate, an intermediate in the TCA cycle, as a carbon source, stimulates vesicle and phialide production. The final stage in conidiation, the conidial development on the phialides, is realised by transferring the cells to a fresh medium containing glucose and nitrate. This medium replacement system allows the study of conidiation in morphologically and biochemically distinct phases (Deans, 1978).

The limitations of these culture techniques arise from the fact that vegetative mycelial growth precedes conidiation, inevitably resulting in a degree of heterogeneity. The relatively recent discovery of a novel growth form in A. niger known as microcycle conidiation (Anderson & Smith, 1971a,b;1972) tends to eliminate such heterogeneity. Microcycle conidiation has been described as the recapitulation of conidiation following spore germination, but without a period of mycelial growth (Smith et al., 1977). The normal asexual reproduction cycle for A. niger illustrated in Fig 3.1 may be compared with that for microcycle conidiation illustrated in Fig 3.2. This phenomenon is known to occur in other fungal species such as Penicillium urticae (Sekiguchi et al., 1975 a; b and c) and Paecilomyces varioti (Anderson et al., 1978).

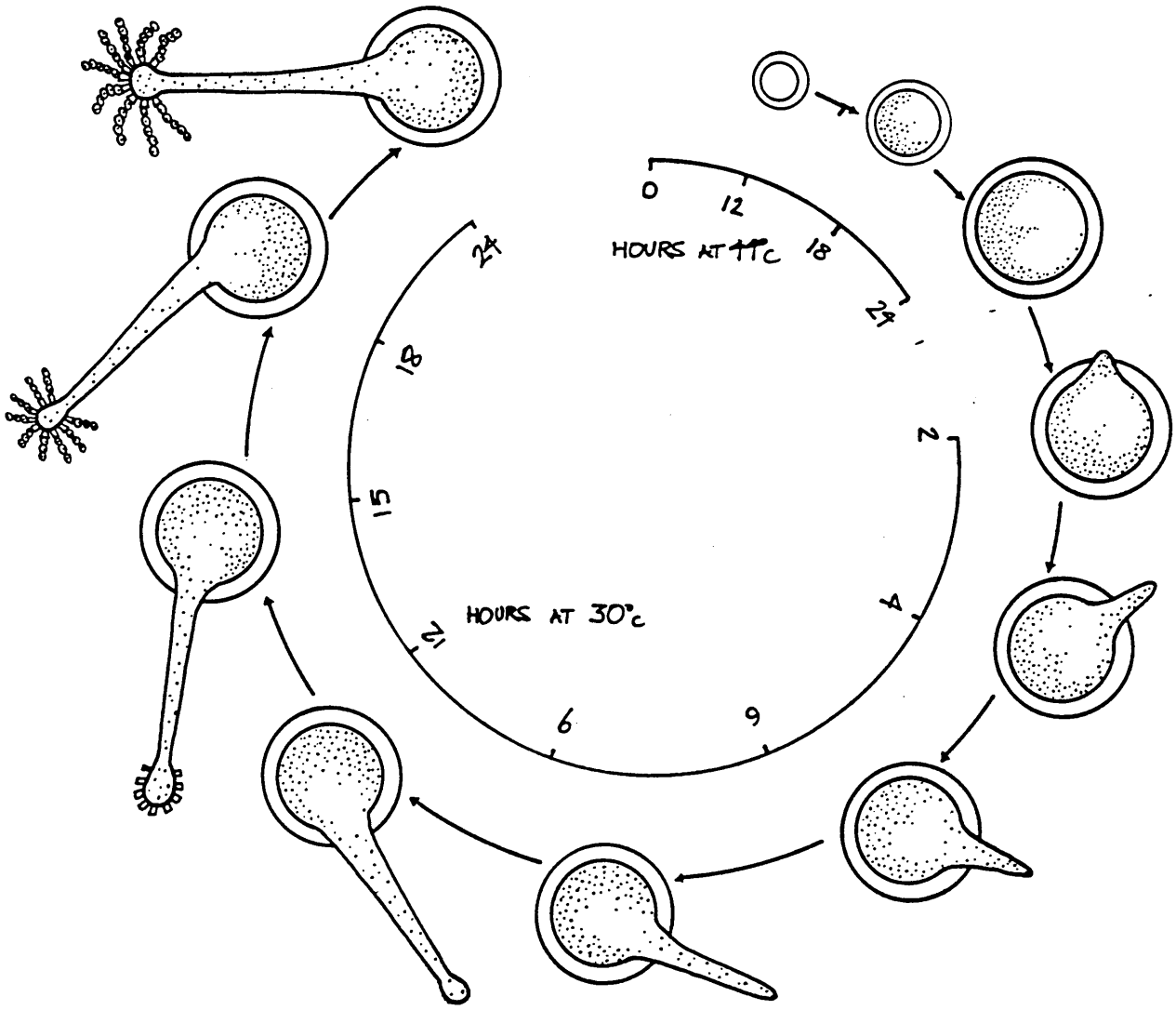


Fig. 3.2 Microcycle conidiation in A. niger (after Smith & Pateman, 1977 reproduced by kind permission of Academic Press).

Limited studies have also demonstrated microcycle conidiation in Aspergillus flavus, Aspergillus giganteus, Aspergillus oryzae and Aspergillus glaucus, and giant cell formation in Rhizopus stolonifer (Smith et al., 1981a). It is also interesting to note that microcycle macroconidiation (Cortat & Turian, 1974) and microcycle microconidiation (Rossier et al, 1977) occurs in Neurospora crassa.

A feature common to all the microcycle conidiation systems is the enlargement of spores by spherical growth at an elevated temperature. All the systems, apart from microcycle microconidiation in Neurospora crassa, use a medium composition which, at non-elevated temperatures, would allow normal vegetative mycelial growth. Under large volume fermenter conditions, a 5% carbon dioxide in air supply is added to the aeration input of the A. niger culture as its absence leads to vegetative germination during the latter stages of giant cell formation at the elevated temperature. The exact role carbon dioxide plays in the microcycle conidiation of A. niger is not fully understood, but studies suggest that it is morphogenetically important (Smith et al., 1981b). However in small scale shake tube culture there is no requirement for an additional carbon dioxide supply to obtain complete microcycle conidiation.

Microcycle conidiation in A. niger is illustrated in Fig 3.2. The incubation of conidia at an elevated temperature causes enlargement by spherical growth producing giant cells. This is the vegetative part of the cycle, and the whole conidiophore structure arises from a single giant cell. Spherical growth occurs for 24h during which the spore increases in size from approximately 3.5 μm to 20 - 25 μm in diameter (Anderson & Smith, 1971a;b).

Such swelling may be obtained by shake tube incubation for 24h at 44°C or a greater biomass can be produced in a fermenter system with a temperature regime of 41°C for 11h, followed by 13h at 44°C.

The original spore contains a single haploid nucleus (Raper & Fennell, 1965) but during spherical growth extensive nuclear division takes place resulting in multinucleate giant cells (Smith et al., 1976). The high temperatures appear to inhibit apical growth which normally leads to hyphal development. However, growth occurs in the form of uniform wall deposition resulting in giant cells with thick cell walls of approximately 2 µm (Deans, 1976).

A temperature reduction to 30°C after 24h results in the resumption of normal apical growth and the onset of conidiogenesis. The conidiophore arises directly from the giant cell which appears to have a similar function to the foot cell of a normal hypha (Anderson & Smith, 1972; Smith et al., 1971). A thick conidiophore stalk is produced which is characteristic of microcycle systems of A. niger and other Aspergilli. All other organisms in which microcycle conidiation has been demonstrated produce conidiophores which have a similar structure to normal germ tubes. The conidiophores of A. niger are much smaller than their subaerial counterparts, the most marked difference being in length, the subaerial being more than five times longer than the submerged. The microcycle conidiophores also differ in that only phialides are produced as opposed to both metulae and phialides (primary and secondary sterigmata) in the normal subaerial forms. On ageing, the conidiophores may form septa, a phenomenom which rarely occurs in the normal subaerial forms.

Microcycle conidia are similar to those of normal asexual development but lack any black pigmentation. They are capable of undergoing microcycle conidiation or reverting to normal mycelial growth depending on environmental conditions (Smith et al., 1981b).

3.2 RESULTS

3.2.1 A. niger Microcycle Growth - Morphological Observations

Giant cell growth followed by conidiogenesis was recorded by photography at various stages during microcycle conidiation. The conidia at the time of inoculation were, on average, 3 - 4 μm in diameter increasing to 8 - 16 μm at 12h and to 20 - 25 μm at 24h. Giant cell spherical growth was accompanied by a thickening of the cell wall reaching a maximum of 2 μm at 24h. The extent of spherical growth is illustrated in Plates 1, 2 and 3 which show the dormant spores and the giant cells at 12 and 24h respectively.

Conidiation was initiated following the decrease in temperature from 44°C to 30°C at 24h. The conidiophore was well developed at 36h as illustrated in Plate 4. The phialides and developing conidia can be seen arising from the vesicle. By 48h the new conidia have been released into the surrounding medium and transverse walls appear in the ageing conidiophore (Plate 5). A giant cell normally produces a single conidiophore and although the development of two conidiophores from the same cell was common, up to six were occasionally observed. The form of microcycle conidiation described above was observed in all experiments which produced the results of the biochemical analyses in Chapters 3, 4, 5 and 6 with the exception of Chapter 3 (Section 3.2.2).

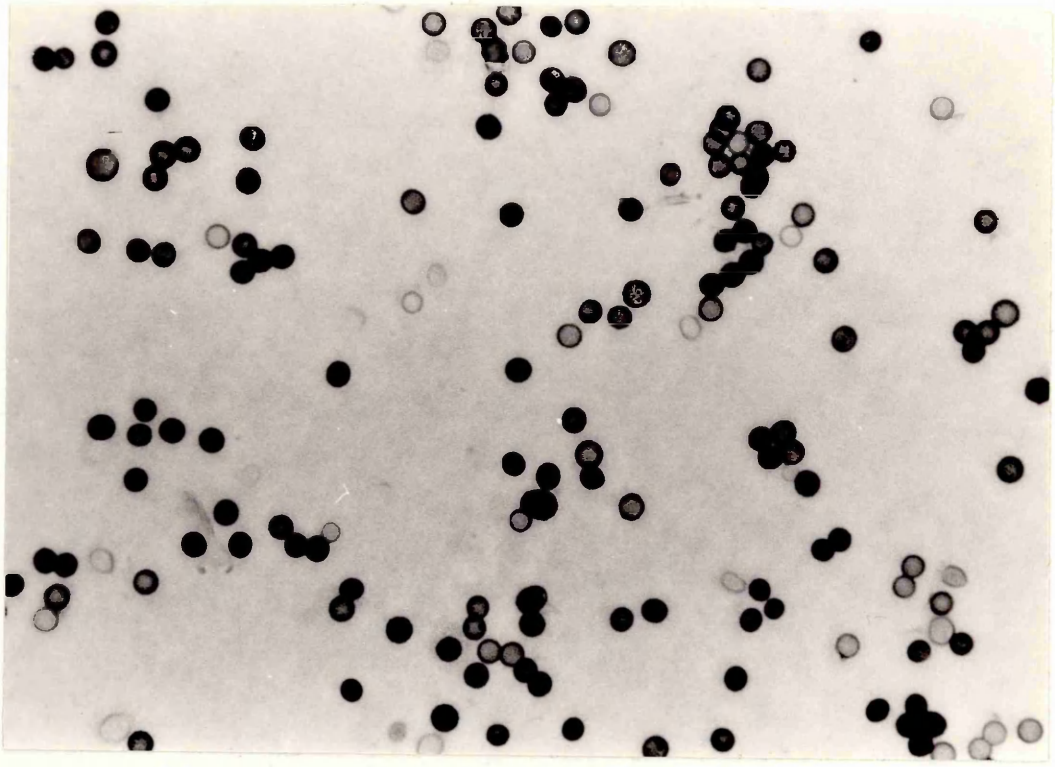


Plate 1. A. niger spores

Magnification x660

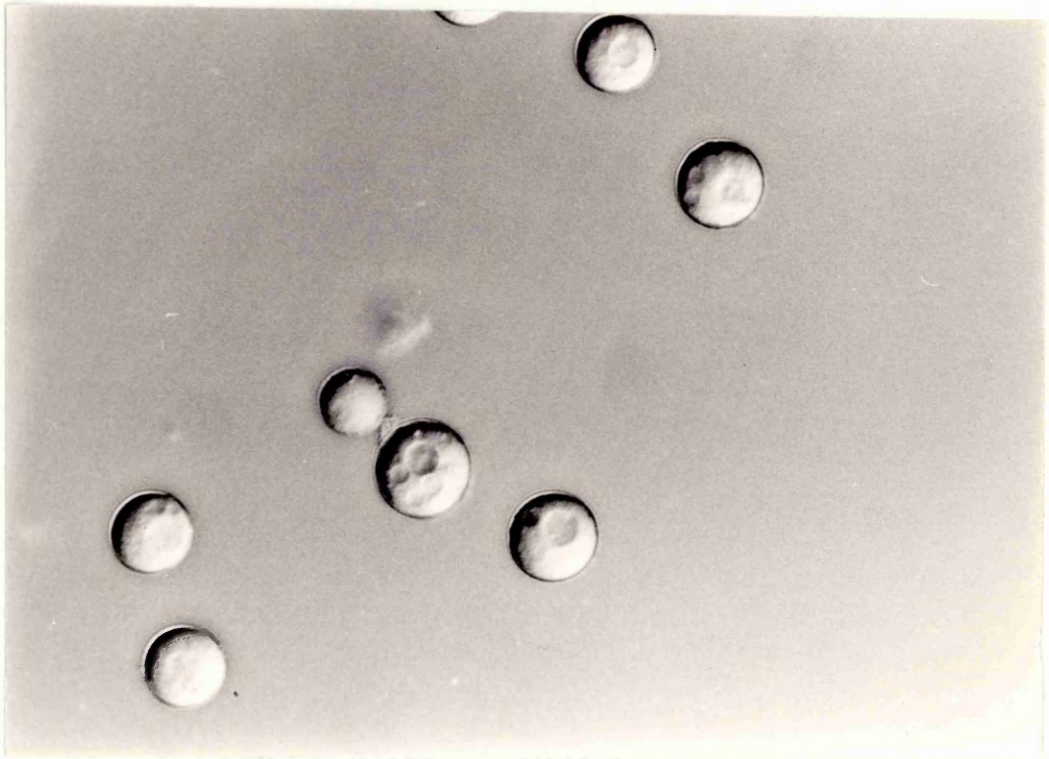


Plate 2. A. niger giant cells at 12h into microcycle conidiation.

Magnification x660

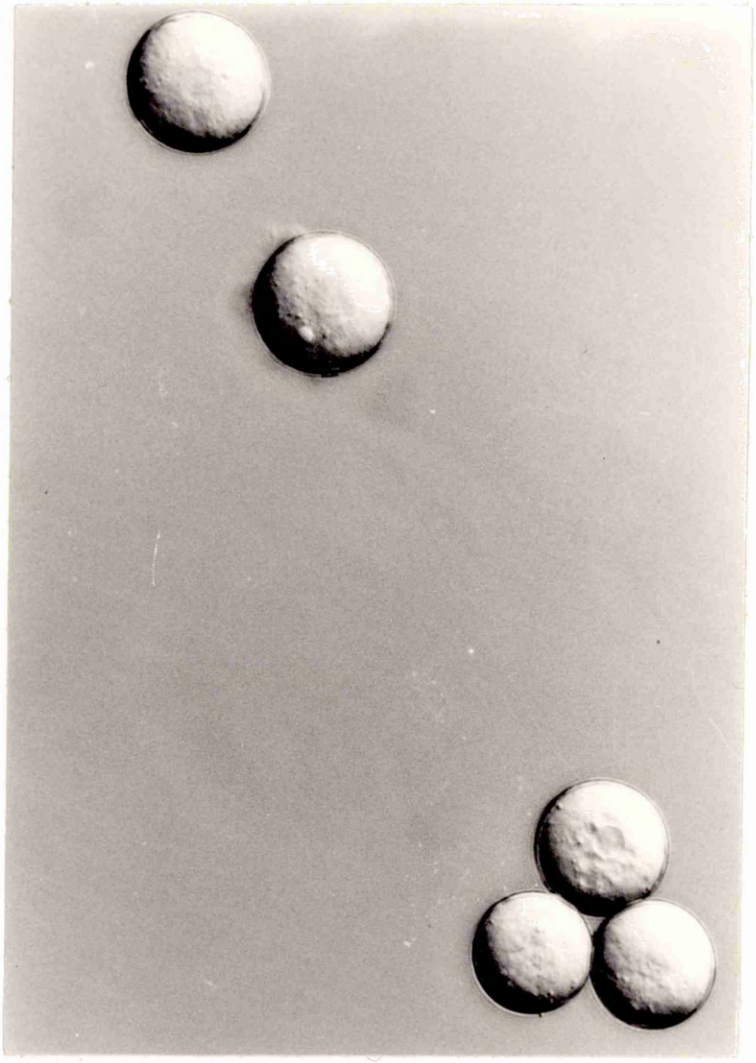


Plate 3. Giant cells of A. niger, 24h into microcycle conidiation. Magnification x660

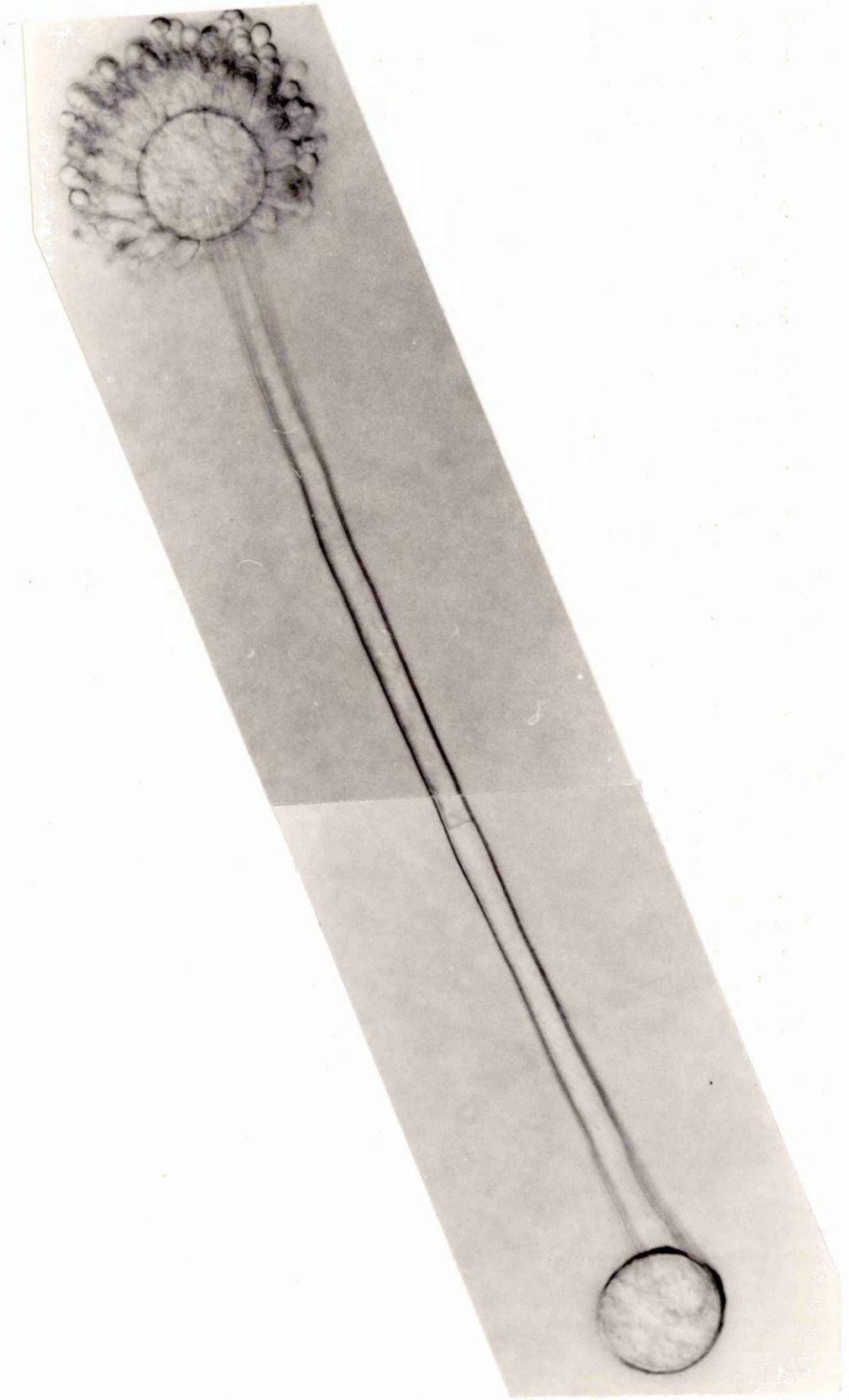


Plate 4. A conidiophore produced by an A. niger giant cell at 36h into microcycle conidiation.

Magnification x660

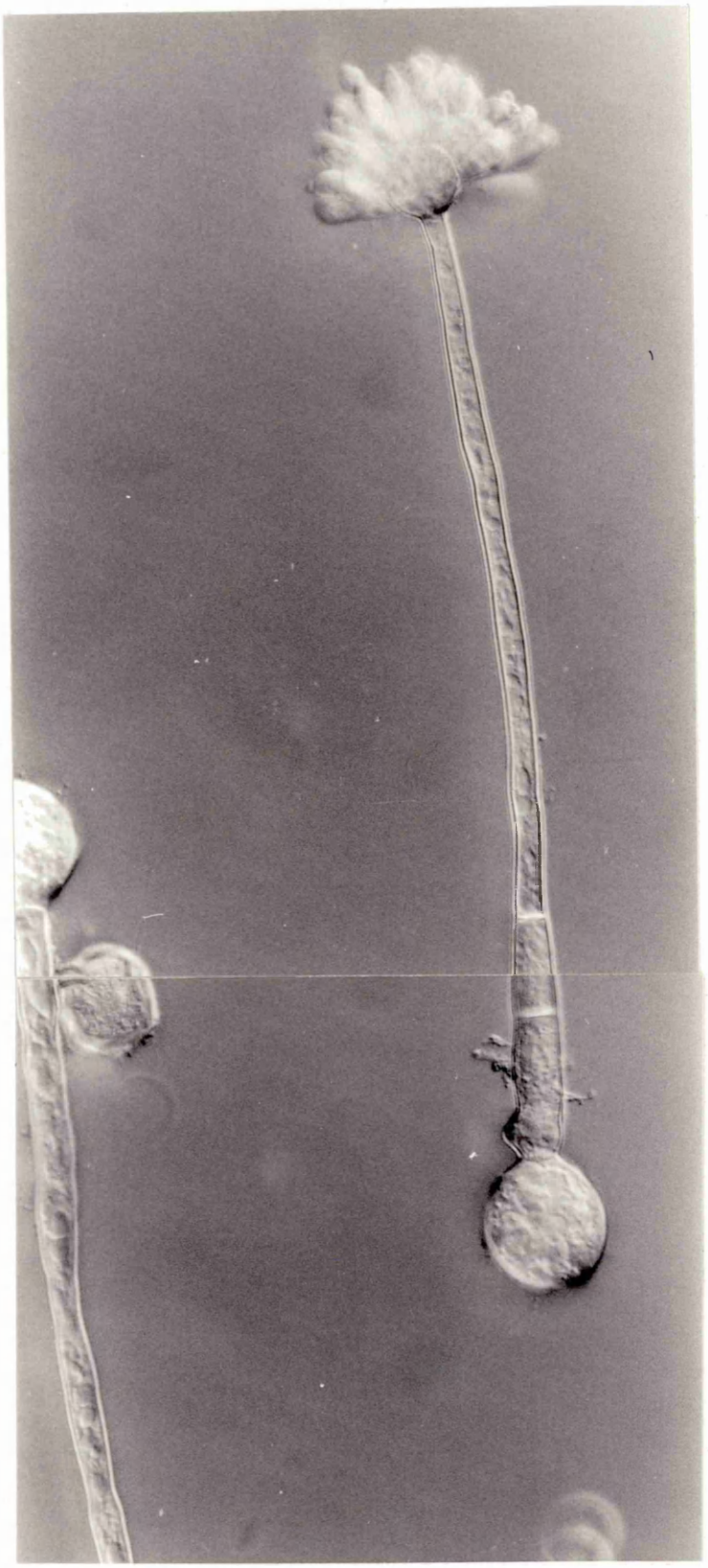


Plate 5. Mature A. niger conidiophore with shed conidia, on completion of microcycle conidiation at 48h.

Magnification x660

3.2.2 Variations in the Growth Pattern during Microcycle Conidiation

Although all parameters during fermenter cultivation of A. niger were carefully maintained, variations in the microcycle pattern of Anderson & Smith (1971a,b) were frequently noted particularly at the latter stages of the study. However, in shake tube cultures little variation in microcycle growth was observed. Investigations were carried out into the causal factors of the aberrant microcycle growth and some changes were made in the parameters used.

(i) Autolysis

In many fermenter cultivations the spores followed the usual pattern of spherical growth to between 11 and 16h but then, during the 44°C temperature regime, autolysis was initiated. Many of the cells had attained the maximum expected diameter of 25 µm at this stage although others were only 8 µm in diameter.

The onset of autolysis was preceded by the occurrence of darker areas which seemed to be in the thickened giant cell wall giving the cell surface a pitted appearance. The cytoplasmic contents were more granular at this stage and this phenomenon is illustrated in Plate 6. The protoplast then shrank away from the cell wall which was still rigid at this point. The cell wall then gradually lost its rigidity and integrity followed by the escape of the cytoplasmic contents from a rupture presumably at a weak point in the wall (Plate 7).

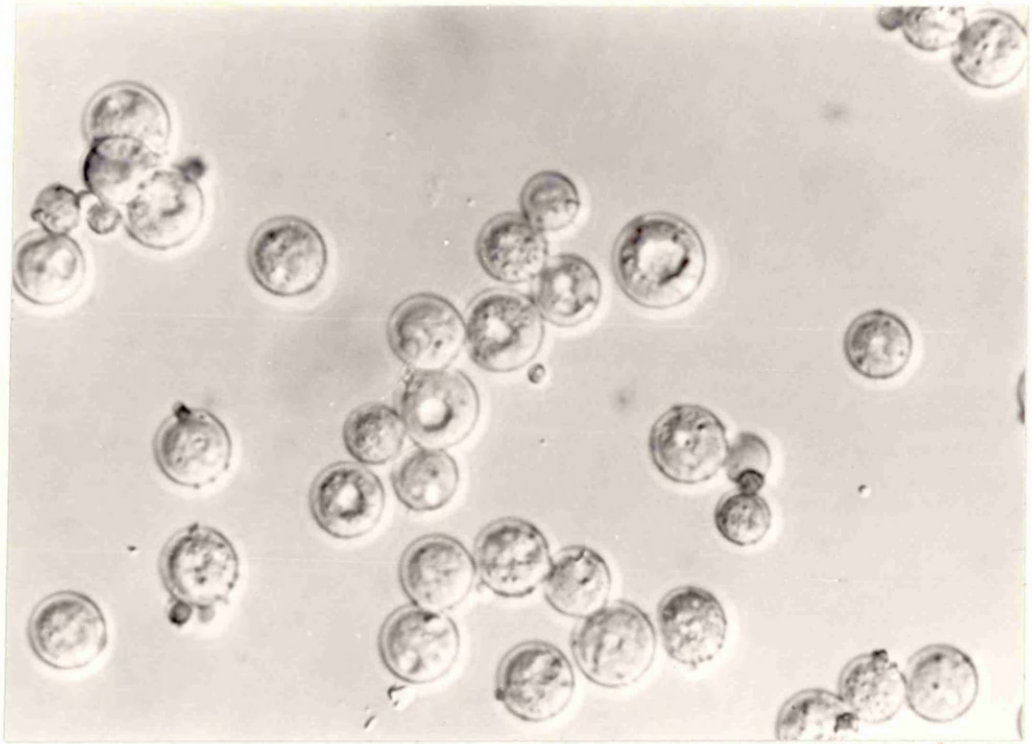


Plate 6. A. niger giant cells, 10h into the microcycle, but about to autolyse. Note granular cytoplasm and 'pits' in the cell wall of some cells.
Magnification x660

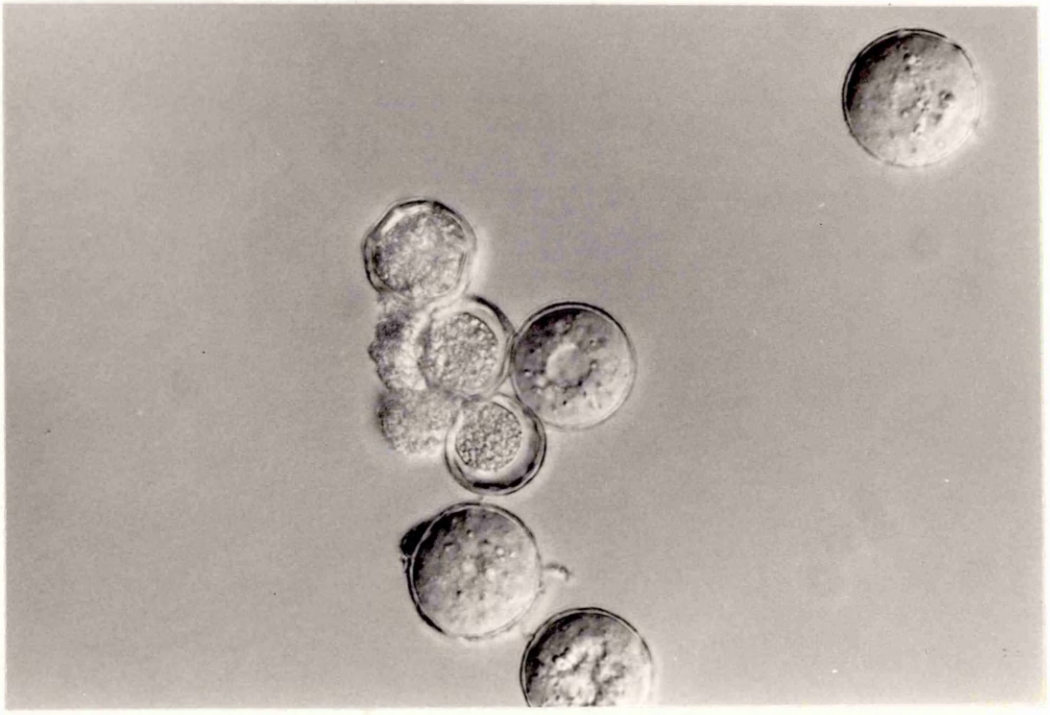


Plate 7. A. niger giant cells at 18h, with some of the cells undergoing autolysis. Note protoplast shrinking from cell wall and loss of cytoplasmic contents.
Magnification x660

In autolysing cultures it was noted that the fermenter growth medium contained much cell debris which gradually disappeared and by 24h at the elevated temperature the medium was completely clear.

(ii) Vegetative Mycelial Growth

During the first 11h at 41°C most cells inoculated into the fermenter began to grow spherically. However, following the increase in temperature to 44°C after 11h, some of the spherically growing cells, having attained cell diameters of 12 - 20 µm, produced vegetative germ tubes, rather than continuing spherical growth. In many cases almost all the cells produced vegetative germ tubes which gave rise to both filamentous and pelleted mycelium, which rarely produced any conidiophores even when the temperature was dropped to 30°C after 24h at the elevated temperatures. Occasionally a conidiophore was observed amidst pelleted mycelial growth but it was difficult to ascertain whether it originated from a mycelium or directly from a giant cell. Filamentous vegetative growth noted at 20h is illustrated in Plate 8 and the pelleted mycelial form observed at 48h in Plate 9.

3.2.3 Modifications to microcycle conidiation conditions

Various attempts were made to increase the efficiency of the fermenter system to produce microcycle conidiation in A. niger in the hope of eliminating the problems of vegetative mycelial growth and autolysis occurring in the 11 - 24h period. The results obtained are summarized in Table 2.1.

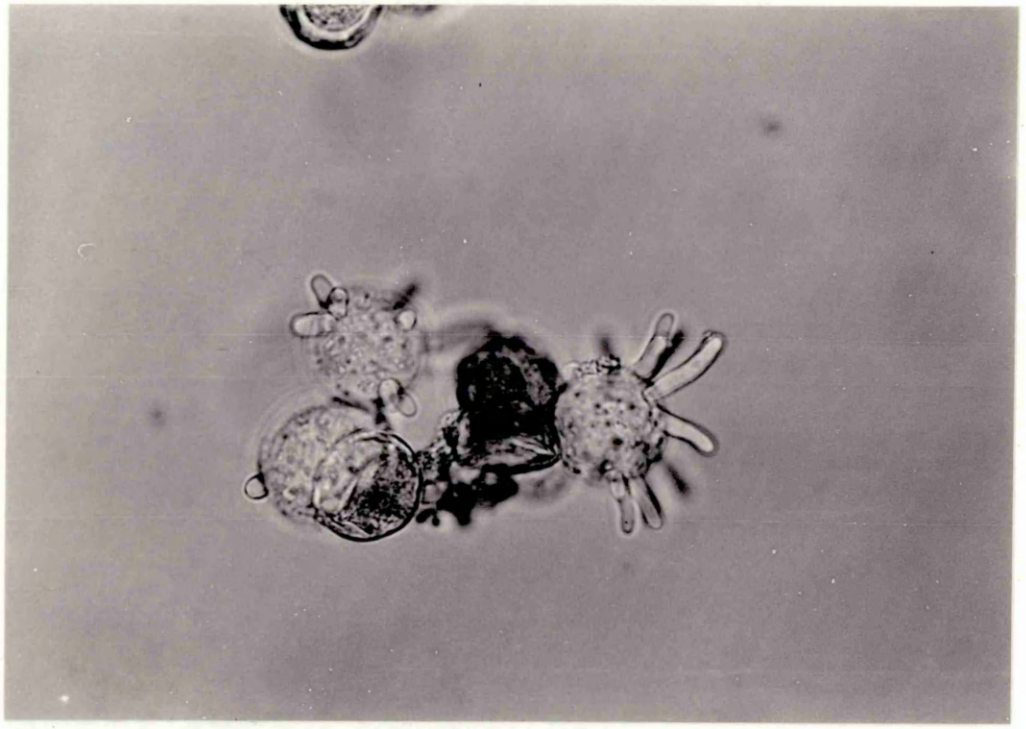


Plate 8. Aberrant A. niger giant cells at 14h grown under microcycle conditions and showing the initial stages of mycelial growth.
Magnification x660

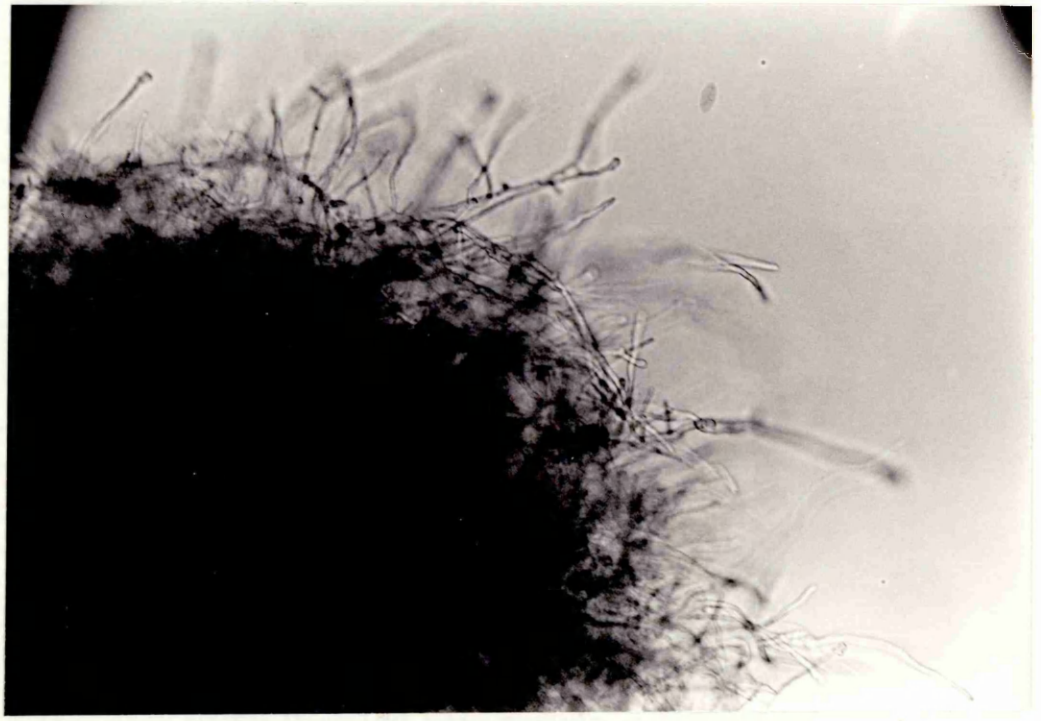


Plate 9. A. niger grown under microcycle conditions for 48h showing a pelleted mycelial form.
Magnification x165

(i) Viability of the conidia

It has been reported that washing the spores five times in distilled water prior to inoculation yields the most synchronised population of cells during spherical growth (Deans, 1978). This procedure was adopted as standard for all microcycle conidiation experiments throughout the study.

Conidia from batches which underwent autolysis and/or vegetative mycelial growth in the fermenter, were tested for the ability to undergo microcycle conidiation in small scale shake tube culture conditions. Without exception microcycle conidiation was achieved.

(ii) Inoculum size

It was observed that the giant cells which autolysed following the increase in temperature from 41^{o}C to 44^{o}C had already swollen to a considerable size by 11h. The diameter of the swollen cells at this juncture was equivalent to that achieved by the giant cells undergoing normal microcycle conidiation at 24h, i.e. 20 - 25 µm.

Preliminary shake tube incubations suggested that an increase in inoculum size decreased the rate of giant cell spherical growth. Since a suggested cause of cell autolysis in the later stages of fermenter cultivations was the rapidity of spherical growth, the inoculum size was increased and the effect on giant cell formation observed.

Doubling the inoculum of conidia resulted in spherical giant cell growth to 12h. Some autolysis occurred in the period up to 18h by which time the surviving cells had a diameter of 20 - 25 μm. By 24h all the cells had autolysed. Halving the conidial inoculum resulted in spherical giant cell growth to 12h, followed by the onset of autolysis.

(iii) Carbon and Nitrogen Sources

An increase in the amounts of the carbon source (glucose) and in the nitrogen sources, glutamic acid (monosodium salt) and alanine were tested in separate experiments for their effect on the microcycle conidiation process. A doubling of the amount of the available carbon resulted in mycelial germination after an initial period of spherical growth up to 11h. A two-fold increase in the glutamic acid in the medium produced spherical growth to 11h followed by mycelial growth for most cells but with some cells remaining as giant cells through the entire 48h growth period. The doubling of the amount of ammonium sulphate supplied in the growth medium also resulted in mycelial growth following an initial 11h period of spherical growth.

(iv) Culture Medium Volume

A decrease in the working volume of the fermenter cultivation system was also tested for its ability to sustain the complete microcycle.

The volume was decreased from 800ml to 500ml of normal culture growth medium. The conidia developed by spherical growth and allowed diameters in the range 10 - 22 μm by 11h. However, during the subsequent 13h at the elevated temperature of 44°C, each giant cell either autolysed or produced mycelial growth.

(v) Stock culture medium

The potato glucose agar used to sustain stock cultures of A. niger was replaced by potato carrot agar in an attempt to eradicate the possibility of attenuation. The potato carrot agar sustained similar mycelial growth to that obtained using potato glucose agar. A mycelial mat with sub-aerial conidiophores was obtained on the potato carrot agar slopes within 6 days as opposed to an average of 4 days with potato glucose agar. Spores harvested from the potato carrot agar slopes, when inoculated into the fermenter system set up for microcycle conidiation growth grew spherically for 11h at 41°C. However, in common with the spores obtained from stock cultures grown on potato glucose agar towards the end of the study, they proceeded to autolyse or produce mycelial growth from 11h onwards.

(vi) Modifications in temperature control mechanisms

Temperature is the most important factor in suppressing normal mycelial growth during A. niger microcycle conidiation (Smith, 1978). It is important to eliminate any small fluctuation in temperature, particularly during the first 11h at 41°C.

To this end, the Gallenkamp temperature control system initially used was replaced by the more sensitive Tecam TE-7 tempette proportional temperature controller. Such a system afforded both internal and external temperature equilibration for the culture vessel, so minimizing the effect of ambient temperature fluctuations. The whole fermenter culture vessel was placed in a constant water bath and a mercury thermometer was placed in the vessel as usual to directly record the temperature of the growth medium. Slight variations in the temperature regime were then tested for their effect on microcycle conidiation. In some experiments using the normal temperature regime of 41°C for 11h followed by 44°C for 13h and 24h at 30°C the giant cells attained a greater diameter at 12h than previously described (Anderson & Smith, 1971a), and microcycle conidiation was complete by 36h as opposed to 48h. Shorter incubation times of 41°C for 10h followed by 3 or 6h at 44°C led to a prominence of mycelial growth with some degree of autolysis occurring.

(vii) Variations in Carbon Dioxide Supply

The ability of the air/carbon dioxide mixing unit to maintain a constant flow of 200ml/min of 5% carbon dioxide throughout the 48h of the microcycle was tested regularly. Fluctuations in the carbon dioxide levels were observed from time to time in both successful and unsuccessful microcycle conidiation cultures. These fluctuations were eliminated when the mixing system was replaced by a ready-mixed 5% carbon dioxide cylinder supply connected to the fermenter culture via a flow meter regulating the supply at 200ml/min.

Incubation time	11 - 13h	18 - 24h
Variation in parameter		
Conidial inoculum x 2	Cell diameter 12-20 µm Some unswollen conidia	90% cells autolysed
Decrease in conidial inoculum x 0.7	Cell diameter 12-19 µm	50% cells autolysed 50% cells producing mycelial growth
Decrease in fermenter working volume to 500ml (visual spore inoculum of 800 x 10^6 conidia)	Cell diameter 11-20 µm	Cells either autolysed or producing mycelial growth
Increase in glucose content of medium x 2	Cell diameter 16-20 µm Some autolysis	50% cells autolysed 50% cells producing mycelial growth
Increase in glutamate and alanine content of medium x 2	Cell diameter 16-20 µm	Cells either autolysed or produced mycelial growth
Temperature of 41°C for 10h, then 44°C for 3h, followed by reduction to 30°C	17 - 20 µm	50% cells autolysed 25% cells producing mycelial growth. 25% giant cells with prominent vacuoles
Temperature of 41°C for 10h, then 44°C for 3h	Cell diameter 16-20 µm Most cells producing mycelial growth	Clumped mycelial growth
Temperature of 41°C for 11h, 44°C for 6h, then reduction to 30°C	Cell diameter 12-19 µm Some cells autolysed	Some autolysis, but majority of cells developing mycelial growth

Table 3.1 The effect of varying parameters in A.niger fermenter cultivations with a view to increasing the efficiency of the system in producing microcycle conidiation.

(viii) Culture vessel contents agitation

The magnetically controlled impellar system in the fermenter was replaced by an overhead propulsion stirring mechanism which afforded greater and more reliable agitation of the culture.

(ix) The ability of other strains of A. niger to undergo microcycle conidiation

Two other strains of A. niger, IMI 50565 and IMI 96215 obtained from the Commonwealth Mycological Institute, Kew, Surrey, England, were tested for their ability to undergo microcycle conidiation. The strains were sub-cultured, the spores harvested and inoculated into shake tube cultures as previously described in Chapter 2. However, both strains, and in particular IMI 50565, had a slower growth rate on potato glucose agar than did A. niger, Strathclyde strain.

The IMI 50565 strain produced spherical growth up to a maximum diameter of 20 μm at 24h, although by this stage, many of the cells had started germinating to produce mycelia. The mycelial growth was mainly clumped despite vigorous agitation. Some cells had autolysed by 24h, others remained unswollen.

The IMI 96215 strain produced cells which had swollen to diameters of 12 - 20 μm by 24h but in the majority of cases mycelial production had been initiated by this stage. The few giant cells which did not produce germ tubes developed conidiophores following the decrease in temperature to 30^{o}C at 24h.

Some cells gave rise directly to conidiophores, but many formed branched mycelial growth from which the conidiophores emerged.

The IMI 50565 strain had developed dense clumps of hyphal growth by 48h with a few conidiophores present. The conidiophores had a very swollen vesicle but small phialides when compared to the A. niger, Strathclyde strain. The IMI 96215 strain produced very swollen vesicles and larger phialides. The growth forms produced by the IMI 96215 strain are illustrated in Plates 10 and 11.

Synchronous growth was not achieved with the two IMI strains tested. Although some cells in both cases have the ability to undergo microcycle conidiation, most germinated to produce mycelial growth even during the period at the elevated temperature of $44^{o}C$.

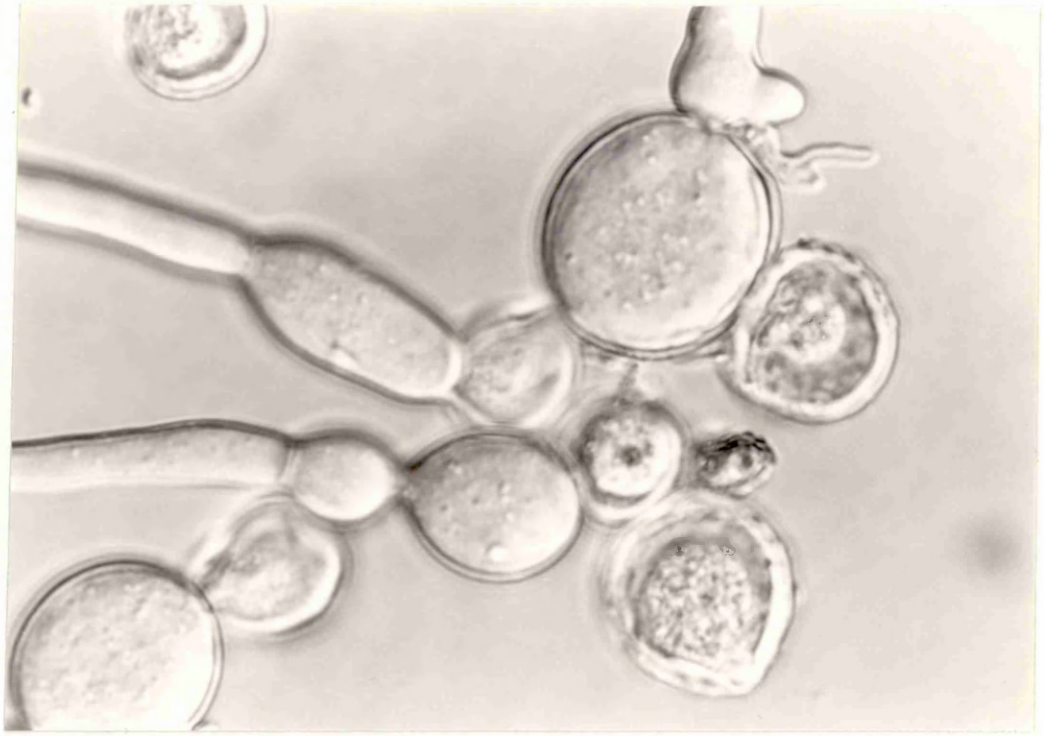


Plate 10. A. niger IMI 96215 after 24h under microcycle growth conditions. Note swollen giant cells, autolysis and mycelial growth.
Magnification x660

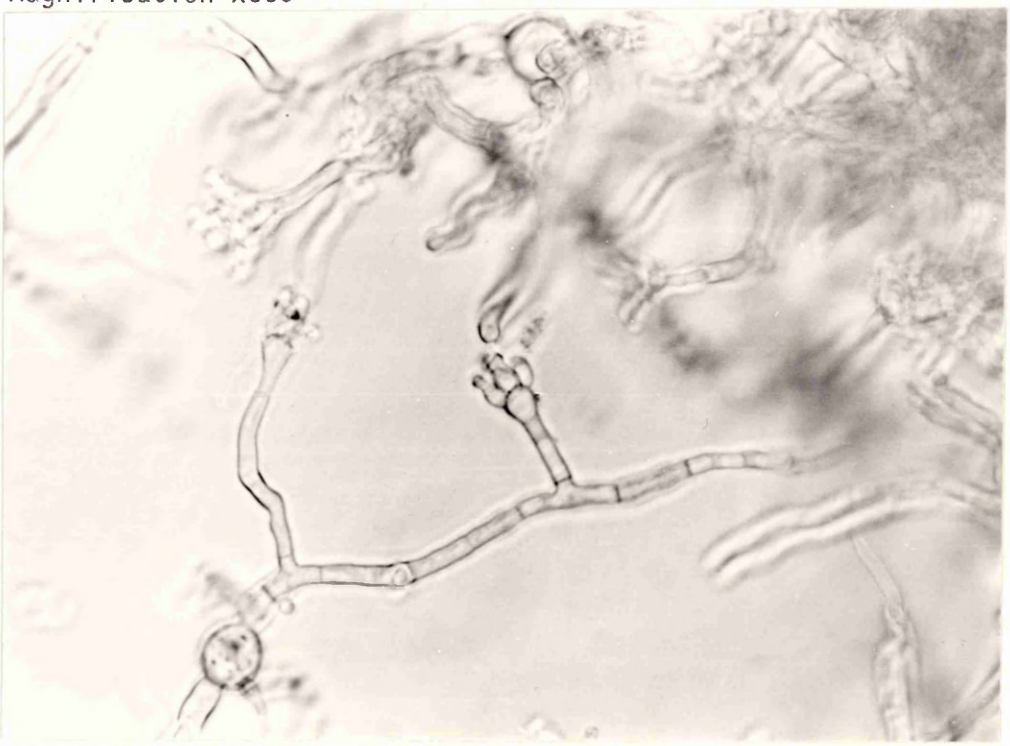


Plate 11. A. niger IMI 96215 after 48h under microcycle growth conditions. Note mycelial growth plus some formation of conidiophores.
Magnification x165

3.3 DISCUSSION

The microcycle conidiation observed is similar in morphological aspects to that described by Anderson & Smith (1971, a;b). However, the microcycle clock appears to have a shorter time duration in the present study, the total cycle being complete by 36 - 38h. An increase in giant cell diameter in comparison to Anderson & Smith's (1971, a;b) observations was recorded at 11h, on occasions the maximum of 25 µm being attained at this stage. At 28h, 4h after the reduction in temperature to 30°C, the conidiophore was elongated. By 32h the swollen vesicle was fully developed and the production of phialides was initiated. By 36h the phialides were mature and conidial development almost complete, at 38h conidia were shed into the surrounding medium. This compares with Anderson & Smith's (1971, a;b) system where the conidiophore vesicle swelling is initiated at approximately 36h and phialide production at 39h. Conidial development occurred between 40h and 48h (Anderson & Smith, 1971;a;b).

The giant cell of *A niger* can produce more than one conidiophore and up to six were observed in some cases. This phenomenon has previously been reported for up to five conidiophores (Smith *et al.*, 1981a).

The observed acceleration of spherical growth in the fermenter may in part explain the difficulties in obtaining conidiophores and conidia from the swollen giant cells. Maintenance of conidia at 41°C for 11h in the fermenter system invariably resulted in spherical growth, but in many cases the subsequent increase in temperature to 44°C caused autolysis or vegetative mycelial growth.

A giant cell diameter of up to 25 µm was attained before autolysis occurred although such dimensions were more usually obtained after 24h at elevated temperatures during normal microcycle conidiation. This can be compared with shake tube culture observations by Anderson & Smith (1971 b) where conidia were incubated for prolonged periods of up to 48h at elevated temperatures and still had the ability to produce conidiophores once the temperature was reduced. In their work, Anderson & Smith (1971 b) noted that after 12h and 24h at 44°C, conidia were able to produce germ tubes at 30°C and some were above to produce conidiophores. After 36h at 44°C most cells could produce germ tubes and conidiophores, with some able to produce conidiophores only. After 48h at 44°C, the cells were unable to produce germ tubes but were able to produce conidiophores (Anderson & Smith, 1971 b).

Carbon dioxide was added to the aeration system in fermenter cultivation as it has been shown to inhibit germ tube formation at 41°C (Kuboye et al., 1976) without retarding spherical growth. Carbon dioxide is known to be a powerful inducer of morphogenetic change in filamentous fungi (Smith & Galbraith, 1971). Its interaction with elevated temperature to induce dimorphic transformations and other spherical cell forms in filamentous fungi has previously been discussed (Anderson & Smith, 1976; Anderson, 1978). Kuboye et al. (1976) further observed that should the temperature of 41°C with a 5% carbon dioxide in air supply, be maintained for longer than 12h, autolysis occurred. An increase in temperature to 44°C before the onset of autolysis prevented this.

In many cases during the present study, rapid spherical growth was achieved for 11h at 41°C, but the temperature increase to 44°C seemed to initiate autolysis. Kuboye et al. (1976) observed that at 41°C spherical growth was more rapid than at 44°C and a better percentage activation of the conidia was achieved.

Giant cell growth involves a rapid cell wall extension of up to 2 µm (Smith et al., 1981 a;b), and may lead to a weakening of the wall structure. In the present study, the giant cells often developed more rapidly than previously described (Anderson & Smith, 1972; Deans, 1978). Also the deceleration in spherical growth which occurred with the increase in temperature to 44°C may occur too late to prevent such weakening. Causal factors may be an inhibition of an essential mechanism in cell wall synthesis or may be due to the increase in lytic activity associated with wall expansion.

Autolysis is normally considered the final stage of cultural development, the main cause being a material imbalance in the fungal hyphae, caused by either internal or external factors. These could be accumulation of toxic metabolites, disturbance of organelles or physical, chemical and enzyme influences which change the intracellular structure of the cell wall. Autolysis often occurs in filamentous fungi due to an exhaustion of, or a deficiency in a medium constituent, particularly carbon (Smith, 1978). An autolytic phase in the A. niger microcycle was reported, by Kuboye et al. (1976), to be due to glucose exhaustion. Nitrogen starvation would render the giant cells unable to produce conidiophores as glutamic acid is known to play an important role at the onset of conidiogenesis (Anderson & Smith, 1971b).

Trinci & Righelato (1970) observed that autolysis does not proceed synchronously in starved mycelial filaments of Penicillium chrysogenum, but occurs in individual compartments. The living parts of the mycelia are able to use the low molecular weight compounds released by lysis as substrates for their growth as demonstrated in Nectria galligena (Lahoz, Beltrá and Ballesteros, 1970) and in Aspergillus (Behr, 1930). Intrahyphal mycelia have also been observed in autolysed compartments of Penicillium chrysogenum (Trinci & Righelato, 1970).

Thus some species use autolysis as a means of survival under conditions of starvation. However, in A. niger fermenter cultivations there is still an ample supply of carbon and nitrogen sources in the medium at the onset of autolysis. Also, the vegetative growth form is a single cellular unit. In effect, autolysis results in the destruction of the entire vegetative structure and the whole genome and cannot therefore be regarded as a means of survival, as in the mycelial growth form.

Some fermenter cultivations resulted in giant cell growth during the first 11h at 41^{o}C, but an increase in temperature to 44^{o}C resulted in mycelial growth. Previous workers (Anderson & Smith 1971b; Kuboye et al, 1976) have reported that a temperature of 44^{o}C inhibits germ tube formation and that the presence of carbon dioxide has a further inhibitory effect. The causal factors in encouraging mycelial growth as opposed to microcycle conidiation were therefore suspected to be inefficient temperature control and/or carbon dioxide availability.

However, even after changing to a more temperature sensitive heating system and supplying a more accurately measured quantity of carbon dioxide to the aeration system together with improved agitation to ensure a gas supply to all cells, mycelial growth and giant cell autolysis continued to occur in some microcycle experiments.

4. LIPIDS OF ASPERGILLUS NIGER DURING MICROCYCLE CONIDIATION

4.1 INTRODUCTION

4.1.1 Fungal Lipid Research - An Overview

The term 'lipid' has traditionally been used to describe a chemically heterogeneous group of substances including fatty acids, fatty acid derivatives, steroids, terpenes and carotenoids. Lipids have in common their solubility in organic solvents such as chloroform, methanol or hexane but are mainly insoluble in water. A few lipids have limited solubility in water which is dependent on polar groups linked to the hydrophobic part of the molecules. Most lipids contain carbon, hydrogen and oxygen. Some contain only the first two elements. Other elements such as phosphorous, nitrogen and/or sulphur, may also be present.

Much of the early work in fungal lipid research was initiated with the objective of using fat as a food material for the increasing world population. In 1870 it was reported that Claviceps purpurea contained 30% fat (see Weete, 1980) and much of the research during the subsequent fifty years was concerned with increasing the fat yield in fungal cultures and defining optimal culture conditions for lipid production and accumulation.

The quest for alternative sources of protein and fat to supplement the world's agricultural production led to the screening of various micro-organisms for their ability to accumulate nutrients. Micro-organisms have a high rate of metabolism, can utilise a variety of substrates, need only a limited space for cultivation, reach maximum nutrient yield within a relatively short time and thus have low production costs.

The problem of food production is becoming more acute with an ever-expanding population, particularly in the third world. India, for example, is one of the major oil seed producing countries in the world, yet the per capita consumption of lipid is only 25% of man's nutritional requirements and even this is deficient in unsaturated fatty acids (Chahal et al., 1979). The lipid industry in Egypt suffers from a shortage of oils and the alternative of using fungi as a source of lipids instead of relying on classical oil crops, is being considered (Farag et al., 1981). In particular, Farag et al. have considered using some fungi to produce arachidonic acid, one of the essential fatty acids in the human diet and the precursor of prostaglandins.

Microbes can convert the cheap forms of carbohydrates (e.g. molasses), which may be readily available in the third world, to fats (Woodbine et al., 1951). Careful consideration must be given to the selection of fungi used in the food industry as many produce mycotoxins. However, such by-products can be used to advantage in the pharmaceutical industry where new and effective anti-bacterial agents are continuously sought. Currently, with the advent of genetic engineering, greater use of fungi can be envisaged for the production of economically important lipids.

During the early 1950s there was a transition from the study of fungi as fat producers to a period of lipid research. This switch in approach was mainly prompted by the development of improved extraction, chromatographic and identification techniques. Thus, the fatty acid composition of many organisms was studied and as techniques were perfected, individual sterols, phospholipids and other components were identified.

At about this time radioisotopes became available and some tracer study work was initiated which looked at certain biochemical pathways and enzymes associated with the lipids of various microfungi.

A number of filamentous fungi have been classed as oleaginous, that is as being able to accumulate high amounts of fat. These include species of Claviceps, Penicillium, Aspergillus, Mucor, Fusarium and Phycomyces (Woodbine, 1959). For any particular organism, the lipid accumulated depends upon the cultural conditions including the carbon-nitrogen ratio and temperature (Bainbridge et al., 1971). The substrates available also exert a considerable effect on lipid production. Redchits (1980) showed that the lipid accumulated in the mycelium of Aspergillus effusus, Aspergillus oryzae, Aspergillus ochracens, Aspergillus sydowi and Aspergillus niger in submerged growth was higher with a hydrocarbon substrate as compared to a sucrose carbon source.

The fundamental knowledge of lipid biochemistry in fungi was gained using only a few organisms such as Saccharomyces cerevisiae and Neurospora crassa. This is similar to Escherichia coli, being the most extensively studied bacterium and the impression given is that it is a blueprint for all bacteria. However, extensive lipid research has been undertaken in the past twenty years mainly due to an interest in such topics as membrane structure and functions. Such research has revealed an immense diversity in the types and functions of lipids in plants, animals, fungi and bacteria. Certain lipids are unique to a single species of plant or bacterium and can be used in taxanomic classification e.g. 13C fatty acids in Cellulomonas spp. (see Harwood & Russell, 1984).

The amount of lipid present in fungi may vary between different species of the same genus and isolates of the same species grown under identical conditions. Hence the percentage of lipid present is of no importance taxonomically, but fungi can be classified on the basis of their fatty acid composition. All fungi contain palmitic, stearic and unsaturated 18C fatty acids but the lower fungi usually contain γ-linolenic acid whereas the higher fungi contain α-linolenic acid (see Harwood & Russell, 1984). Farag et al., (1981) suggested that the ratio of various fatty acids present could be used to identify fungi of the Aspergillus genus. Also it has been observed that 16:1 and γ-linoleic acids are present in A. niger but not in A. flavus (Shaw, 1965). Studies undertaken by Stretton et al. (1976) showed that a group of 15 Aspergillus species could be related to each other based on the percentages of five fatty acids present. It was noted that A. niger and A. candidus were closely related, confirming the observations of Smith (1969).

4.1.2 Lipid classification and functions

The main lipid class consists of fatty acid moieties linked by an ester bond to an alcohol e.g. glycerol, or by amide bonds to long chain bases (sphingoid bases). Hydrolysis of lipids can yield phosphoric acid, organic bases, sugars or more complex components. A sub-division of lipids can be made on the number of products liberated during hydrolysis. Lipids yielding one or two different types of products on hydrolysis are termed 'simple' whereas those releasing three or more products are labelled 'complex'.

Simple lipids are often termed neutral lipids and complex lipids are usually termed polar lipids.

Lipid nomenclature has developed rather haphazardly and, although various systematic classification schemes exist, trivial names are in common use. The main classes of neutral lipids found in fungi include fatty acids, acylglycerols, methyl, ethyl and sterol esters, aliphatic hydrocarbons, sterols, carotenoids and polyprenols. The polar classes consist predominantly of glycerophospholipids and sphingolipids.

The trivial names used for fatty acids are often named after the source of their first extraction e.g. palmitic acid from palm oil. A systematic naming of fatty acids is based on the longest hydrocarbon chain containing the carboxyl group. The corresponding alkane is incorporated into the fatty acid name and the carbon atoms are usually numbered sequentially beginning at the carboxyl end of the molecule (Δ system). Sometimes the fatty acid carbons are numbered from the methyl end and this system is prefixed by ω. The presence and number of double bonds is indicated by incorporating -ene, diene etc into the name, the position being designated by the number of the carbon atom nearest the carboxyl carbon involved in the ethylenic bond.

A short hand system of fatty acid nomenclature is also widely used. The molecules are designated by two numbers separated by a colon. The first number indicates the number of carbon atoms present and the second the number of double bonds. Additional figures in parenthesis show the double bond positions and configuration, c and t indicating cis and trans respectively.

Examples of fatty acids most frequently found in fungi, along with their alternative names are given - Table 4.1.

Fatty acids are produced by all living organisms and are potentially the most abundant lipid class found in nature. However, the free carboxylic acids generally exist only in very small amounts as they have a great affinity for protein and are readily incorporated into complex lipids.

Fatty acids are sparingly soluble in water and are responsible for the hydrophobic propeties of the complex lipids. They occur as esters of glycerol forming triacylglycerol in particular which functions as a long term storage material which can be used for energy and carbon skeletons during growth and development. Fatty acids are a major source of energy in living organisms and produce twice as much energy per gram on oxidation than either carbohydrates or proteins. Using lipid as a storage material also has the advantage of a zero water requirement whereas stored carbohydrate in the form of glycogen is 73% (w/w) water (Spencer, 1975). In fungi triacylglycerols constitute up to 90% of the total lipid content and can be observed as droplets in the mycelial and spore cytoplasm (Weete, 1980). Fatty acids also occur as esters of sterols, sugars, glycerophosphates, sphingolipid bases (N-acyl) and hydroxy fatty acids. Thioesters (coenzyme A and acyl carrier protein) are the main forms of fatty acids involved in lipid metabolism.

Plants, and to a lesser extent other organisms including fungi produce a wide variety of chemical compounds termed terpenoids, all of which are based on a five carbon building block (isoprene unit) illustrated in Fig. 4.1.

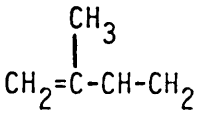
Trivial name	Symbol	Structure	Systematic name
Palmitic acid	16:0	$CH_3(CH_2)_{14}COOH$	hexadecanoic acid
Stearic acid	18:0	$CH_3(CH_2)_7$-$CH_2(CH_2)_7COOH$	octadecanoic acid
Oleic acid	18:1 (9c)	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	cis-9-octadecanoic acid
Linoleic acid	18:2	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	cis,cis-9,12,octadecadienoic acid
α-Linolenic acid	18:3 (9c,12c,15c)	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	all cis-9,12,15-octadecatrienoic acid
γ-Linolenic acid	18:3 (6c,9c,12c)	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_4COOH$	all cis-6,9,12-octadecatrienoic acid

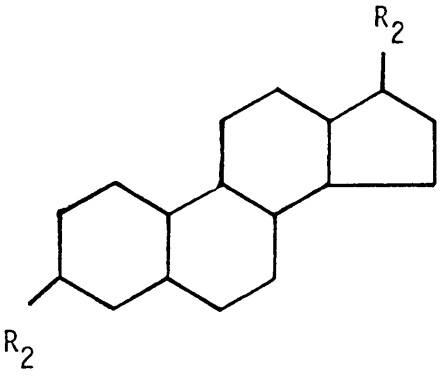
Table 4.1 Examples of fatty acids : their structure and designation.

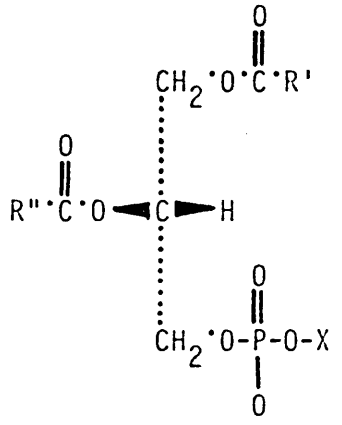
Sterols represent one group of triterpenoids which are derived from squalene, a thirty carbon compound containing a fused four-ring system (see Fig. 4.1). They occur in the free form or esterified with a long chain fatty acid. Yeast is the most primitive organism synthesizing substantial amounts of sterols and they constitute up to 10% of the cellular dry weight (see Harwood & Russell, 1984). Yeasts have been used as a source of sterols for decades and have, along with other fungi, being employed as experimental organisms in elucidating the pathway of sterol biosynthesis. Ergosterol is the main sterol in yeasts, although up to twenty others have been isolated as intermediates in its biosynthesis, or as end products (Parks et al., 1978). Phycomycetes also contain ergosterol as the main sterol representing up to 90% of the sterol content in some species e.g. Mucor rouxii (see Weete, 1980).

Sterols function as permeability regulators in cell membranes acting by altering the internal viscosity and molecular motion of other lipids (Demel & Dekruyff, 1976). They are required for the optimal activity of some enzymes e.g. ATPase (Cobon & Haslam, 1973) and for yeast growth under anaerobic conditions (Weete, 1980). The Pythiaceae are unable to synthesize sterols and although they are able to grow in their absence, have a requirement for them for reproduction (Hendrix, 1970).

Aliphatic hydrocarbons have been reported in fungal spores of many species (Weete, 1980) and the suggested functions are similar to those of the epicuticular wax of higher plants, i.e. the prevention of desiccation, protection from extreme temperatures, resistance to microbial attack, inhibition of germination and playing a role in infection processes.

(i)  an isoprene unit

(ii)  Squalene

(iii)  Glycerophospholipids

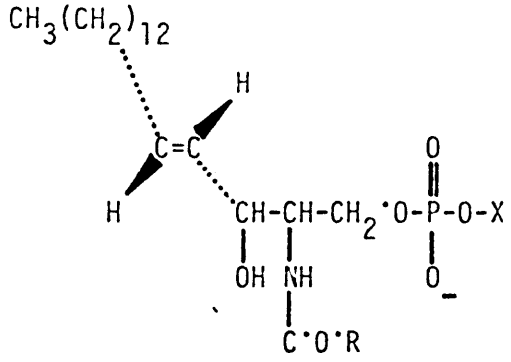
 Sphingophospholipids

Fig 4.1 (i) An isoprene unit, the building block for terpenoids
(ii) Squalene, from which sterols are derived
(iii) The chemical structure of glycerophospholipids and sphingophospholipids

Candida utilis has a pronounced hydrocarbon content in its membranes as compared to other cellular components suggesting a role in membrane structure (Fabre-Joneau et al., 1969). Mycelial hydrocarbons have been reported for three soil fungi, Penicillium sp., Aspergillus sp. and Trichoderma viride (Jones, 1969).

Phospholipids are sub-divided into two large groupings depending on the alcohol with which the fatty acids are esterified. Glycerophospholipids have glycerol as the alcohol, sphingophospholipids have sphingosine. Sphingophospholipids have been reported in small amounts in yeasts and filamentous fungi but their function is uncertain. However certain groups of sphingophospholipids exhibit immunolgical activity and some are components of cell membranes.

A great variety of glycerophospholipids are found in all organisms including fungi and they perform an important structural role in biological membranes. The variation is due to the types of molecules that can be present at the 'X' group and at the acyl group which usually contain long-chain fatty acids. A stereo-specific numbering (sn) system of nomenclature exists for naming phospholipids, but a shorthand method is in common use. The term phosphatidyl is used to denote the phosphatidate portion of the molecule. The two main glycerophospholipids found in fungi are phosphatidylcholine and phosphatidylethanolamine, and lesser amounts of phosphatidylserine, phosphatidylinositol, cardiolipin (diphosphatidylglycerol) and others.

Biological membranes function in protecting the integrity of cells and cellular organelles and in transport processes to and from the external environment as well as within the cell. Membranes

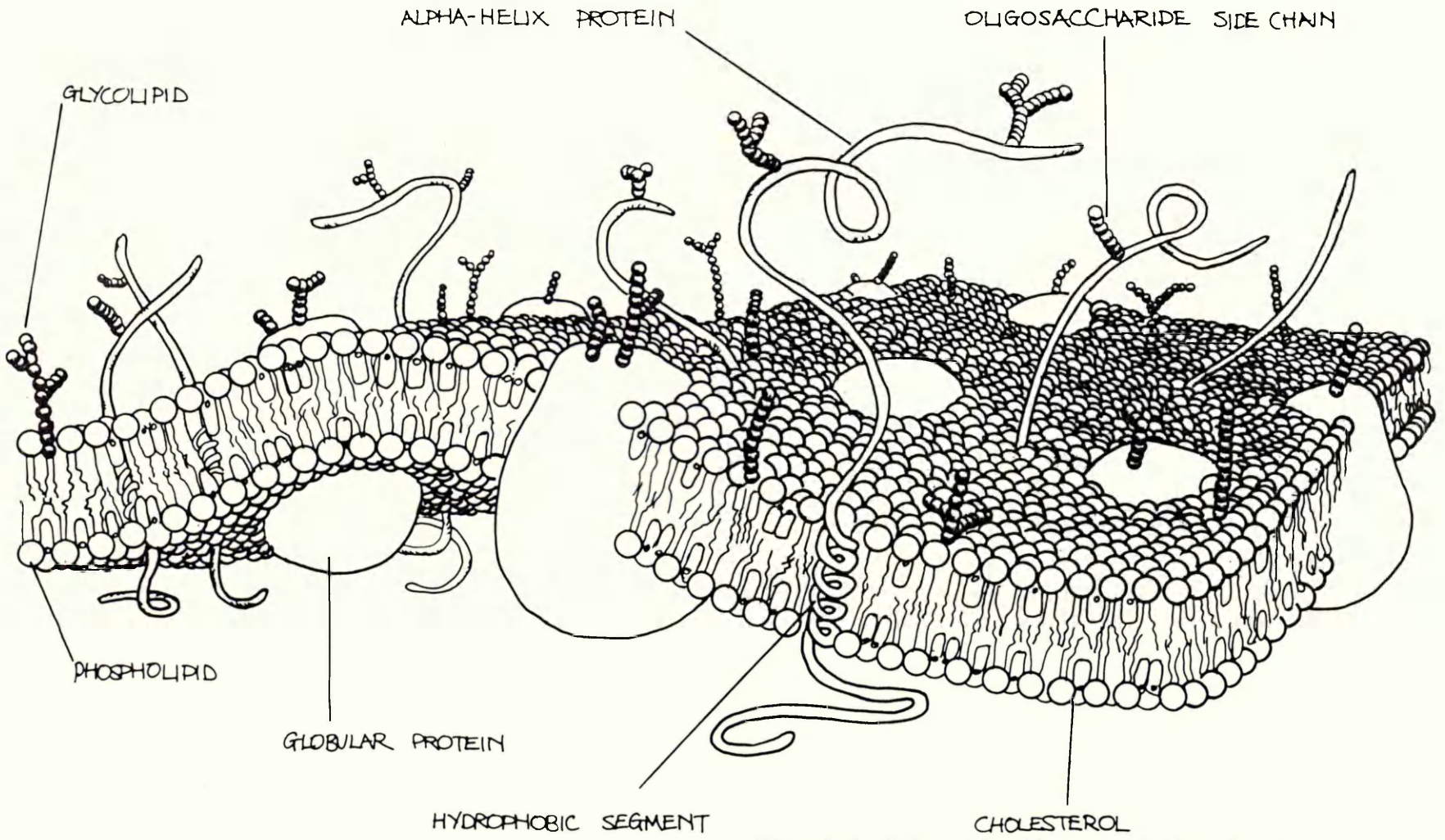


Fig. 4.2 Schematic diagram of the plasma membrane of a cell showing a phospholipid bilayer.

provide environments, often hydrophobic where enzymes and other cellular processes can operate effectively. A considerable proportion of fungal phospholipids are to be found in the main membranes of the cell viz: plasmalemma, vacuolar membrane, endoplasmic reticulum and the mitochondrial membrane. It is known that phospholipids, and in particular phosphatidylcholine are required for the activity of certain membrane-bound fungal enzymes (Coleman, 1973). Lipid and protein are the main constituents of membranes. The current concept is of a fluid lipid bilayer with some proteins (integral) within the membrane while others (peripheral) are attached to the inner surface (see Fig. 4.2).

4.1.3 Study of fungal lipids

Since lipids are complex mixtures of chemical species, progress in lipid research was hindered due to the inefficiency of the normal chemical purification methods. The ability to study a single lipid species was afforded by chromatographic techniques available in the 1950s. The first step in lipid analysis is the separation of the lipid from other products in the tissues. Lipids can be extracted by using various organic solvents, advantage being taken of their low water solubility. As lipids usually exist in an organism as lipoproteins or lipopolysaccharides, the extracting solvents must be sufficiently polar to overcome the forms of attraction in these complexes.

During extraction care must be taken that lipolytic enzymes are de-activated and that lipid recovery is complete. Ideally lipids should be extracted immediately after removal from the living organism, so minimizing the change in components. The complex mixture of individual lipid classes obtained from the extraction procedure can only be separated into the pure constituents by chromatography.

The total lipid present in fungal vegetative hyphae has been widely reported with values of 1 to 56% of the dry weight (see Weete, 1980), depending on the age of the fungus, cultural conditions and fungal species. The lipid content of spores of most fungal species generally range between 5 and 17% of the cellular dry weight (see Weete, 1981). Lipid content may vary with the age of the conidia e.g. conidia from 4 day old cultures of Aspergillus fumigatus contained 6% lipid, but mature conidia from 7 day cultures, 9.9% lipid (Tsukhara, 1980). Rust spores tend to have the highest lipid content and lipid metabolism during germination has mainly been studied in fungi with the greater lipid presence. Respiratory quotient values obtained using fungal spores from several species has revealed that lipid is utilised during germination e.g in the case of rust species (Shu et al., 1954), Fusarium solani (Cochrane et al., 1963), Neurospora crassa (Turian & Bianchi, 1972) and Neurospora sitophila (Owens, 1955). Many spores contain lipid globules but these tend to become reduced in size or disappear during germination, becoming prominent again in sporulating hyphae and developing conidia (see Harwood & Russell, 1984).

Lipid bodies have been reported on freeze-etched replicas of dormant A. niger conidia, and the polar lipid content is higher that the neutral lipid fraction (Gunasekaran et al., 1972c). Glycogen globules are present in A. niger giant cells but are lacking in emerging conidiophores (Deans, 1978). In yeast ascospore germination there is some controversy over the role of lipid as a reserve material. Steele and Miller, (1974) suggest that the disappearance of lipid is too slow for it to be of any significance in spore germination, whereas Rousseau & Halvorson, (1973) report that it is utilised. Saccharomyces cerevisiae has a relatively high neutral lipid content but carbohydrate, mainly glycogen and secondly trehalose are the principal substrates during germination (Sols et al., 1971).

Glycogen is a storage material which is an important constituent of fungal spores, with D-mannitol and trehalose as soluble reserves (Cochrane, 1958). The nature and relative amounts of lipids and other reserve materials may vary with the carbon and nitrogen sources and ratios, and there is also much variation between fungal strains (Lewis & Smith, 1967).

4.1.4 Lipid accumulation during fungal growth and development

Aspergillus nidulans can accumulate very high concentrations of neutral lipids (Snigh & Walker, 1956), but this requires excess hexose and prolonged incubation. Bainbridge et al., (1971) found that although the lipid content of A. nidulans mycelia falls in continuous culture fed a maintenance level of glucose, it rises upon carbon starvation when other cell constituents are used in its synthesis.

Similarly, lipid accumulation occurred in Candida 107 grown on a medium with a high carbon : nitrogen ratio. When the nitrogen is exhausted the cells continue to assimilate the carbon and use it for lipid synthesis. The mechanism of lipid accumulation cannot be attributed to the rate at which glucose enters the cell and it is suggested that the activity or regulation of acetyl CoA carboxylase may be important (Botham & Ratledge, 1978). The total lipid content of A. niger mycelia has been reported by various workers (Woodbine et al., 1951; Chahal et al., 1979). Further information on the lipid classes of A. niger mycelia (Farag et al., 1981) and conidia (Gunasekaran et al., 1972c; Weete & Laseter, 1974) is tabulated in Table 4.2. Some aspects of macromolecular synthesis during microcycle conidiation in A. niger have been studied (Smith et al., 1981) and the total lipid present in the cell wall has been reported (Deans 1978; Smith et al., 1981), but little work has been undertaken with regard to the various lipid classes.

The present study aims to identify any changes in the overall lipid content and in the constituents of the various lipid classes during the processes of giant cell formation and conidiophore production in microcycle conidiation of A. niger. The dry weight and protein content of the A. niger cells undergoing microcycle conidiation was also measured. Thus the lipids and esterase (see Chapter 5) present can be expressed in terms of cellular number, dry weight or protein content.

	% of Total Fatty Acid				
	Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid	Linolenic Acid
Conidia (present study)	16.5	7.3	36.3	36.1	1.4
Conidia (Gunasekaran et al., 1972c)	30.6	5.3	33.3	27.4	-
Mycelia (24h, present study)	25.1	4.7	38.9	27.2	0.2
Mycelia (Weete, 1980)	15.8	7.2	21.3	37.8	15.6
Mycelia Farag et al., 1981)	20.5	15.1	34.5	18.4	-
Mycelia (Stretton et al., 1976)	16.8	0.3	25.7	51.1	1.4

Table 4.2 Fatty acid content of A. niger conidia, mycelia and giant cells

4.2 RESULTS

4.2.1 Dry weight measurements

The dry weight of the A. niger cells throughout microcycle conidiation was measured as described in Chapter 2.12. A marked increase in dry weight was noted over the first four hour period of giant cell formation as illustrated in Fig. 4.3. The spore dry weight was 0.31mg per 1 x 10^6 cells, rising to 1.34mg per 1 x 10^6 cells at 4h. Little change occurred from 4 to 16h although there was possibly a slight decrease in cellular dry weight from 4 to 8h with a weight of 1.18mg per 1 x 10^6 cells recorded at 8h. A slight increase in cellular dry weight possibly occurred from 8 to 12h but was more pronounced in the 12 to 16h period with measurements rising from 1.37 to 1.71mg per 1 x 10^6 cells. From a cellular dry weight of 1.71mg per 1 x 10^6 cells at 16h, a decline over the next four hours to a level of 1.24mg per 1 x 10^6 cells at 20h. This value remained relatively unchanged over the final four hour stage of giant cell formation to 24h.

A marked increase in cellular dry weight was recorded over the 24 to 32h period of conidiophore initiation and elongation. A slight decrease in dry weight from 2.66mg per 1 x 10^6 cells at 32h to 2.26mg per 1 x 10^6 cells at 36h was noted. However there was a subsequent increase in biomass from 36h to 48h. This increase in cellular dry weight was particularly rapid over the final four hours of the microcycle with a biomass of 4.48mg per 1 x 10^6 cells at 44h rising to 10.38mg per 1 x 10^6 cells at 48h.

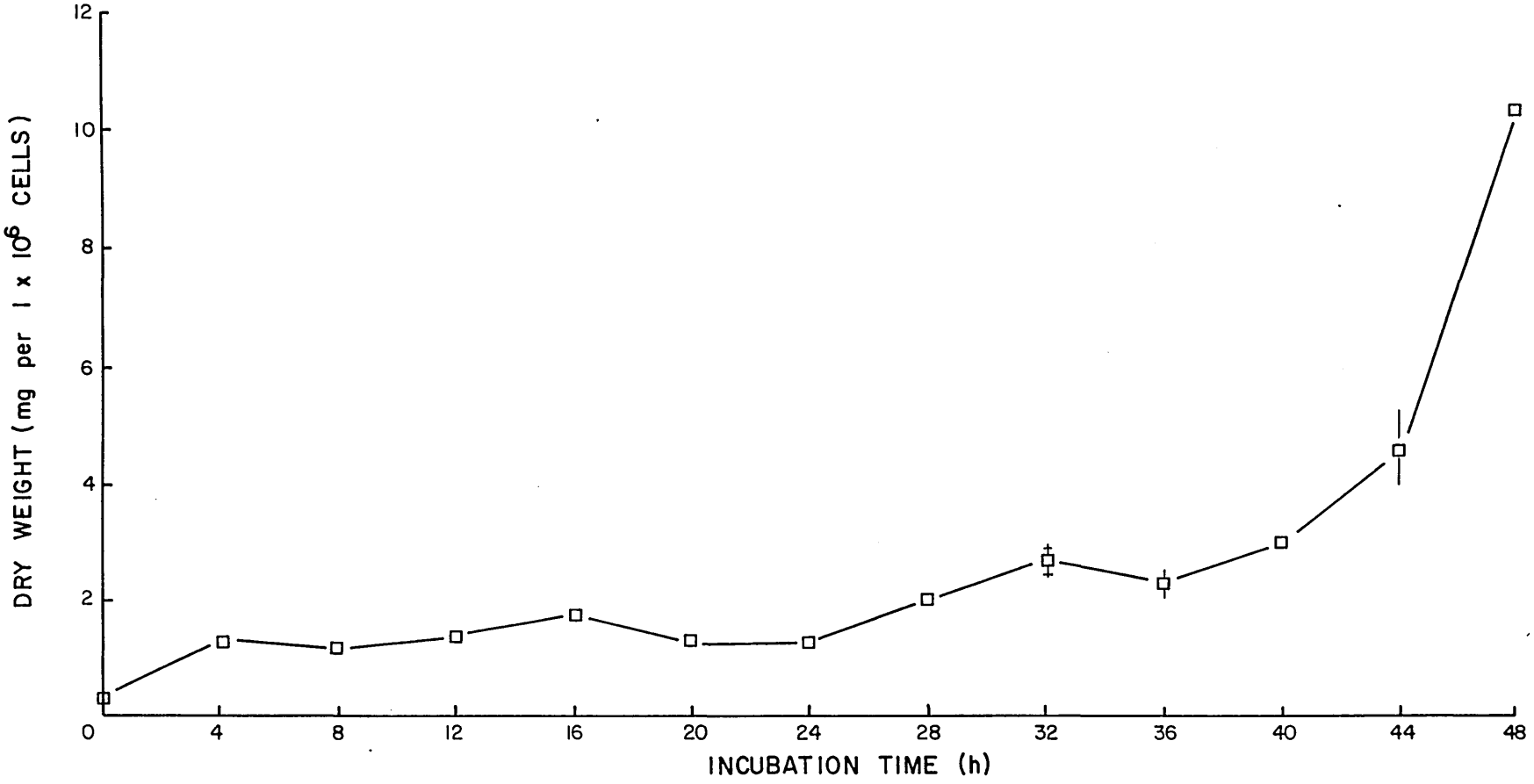


FIG. 4.3 DRY WEIGHT MEASUREMENTS OVER THE 48 h. MICROCYCLE CONIDIATION PERIOD IN A. NIGER.

4.2.2 Cellular Protein Levels

The protein content of the A. niger cells undergoing microcycle conidiation was determined as outlined in Chapter 2.11, and the results illustrated in Fig. 4.4. During spore germination, the cellular protein levels rose slightly from 1.62 µg per 1 x 10^6 spores to 5.05 µg per 1 x 10^6 cells at 4h. From 4h onwards there was a rapid accumulation of protein in the cells, and the giant cells at 24h contained 41.3 µg per 1 x 10^6 cells. However, following the decrease in temperature from 44°C to 30°C at 24h allowing conidiophore initiation, the protein levels possibly dropped slightly to 36.7 µg per 1 x 10^6 cells at 28h. A gradual increase in cellular protein possibly occurred from 28 to 32h, and a more pronounced increase occurred from 32 to 36h with levels reaching 61.7 µg per 1 x 10^6 cells at 36h. A subsequent rapid increase in cellular protein was recorded from 36 to 48h, resulting in 209 µg of protein per 1 x 10^6 cells at 48h.

4.2.3 Total Lipid Content

The total lipid content of A. niger cells was determined over the 48h period of fermenter microcycle growth as described in Chapter 2.7 and 2.8. The results are illustrated in Fig. 4.5 and show an accumulation of lipid throughout the 24h period of giant cell formation. An initial lipid content of 0.34 µg per 1 x 10^6 spores was recorded which rose to 5.0 µg per 1 x 10^6 cells by 4h. From 4h to 20h the rate of lipid accumulation was more gradual with a sharp increase from 9.3 µg at 20h to 33.9 µg per 1 x 10^6 cells at 24h.

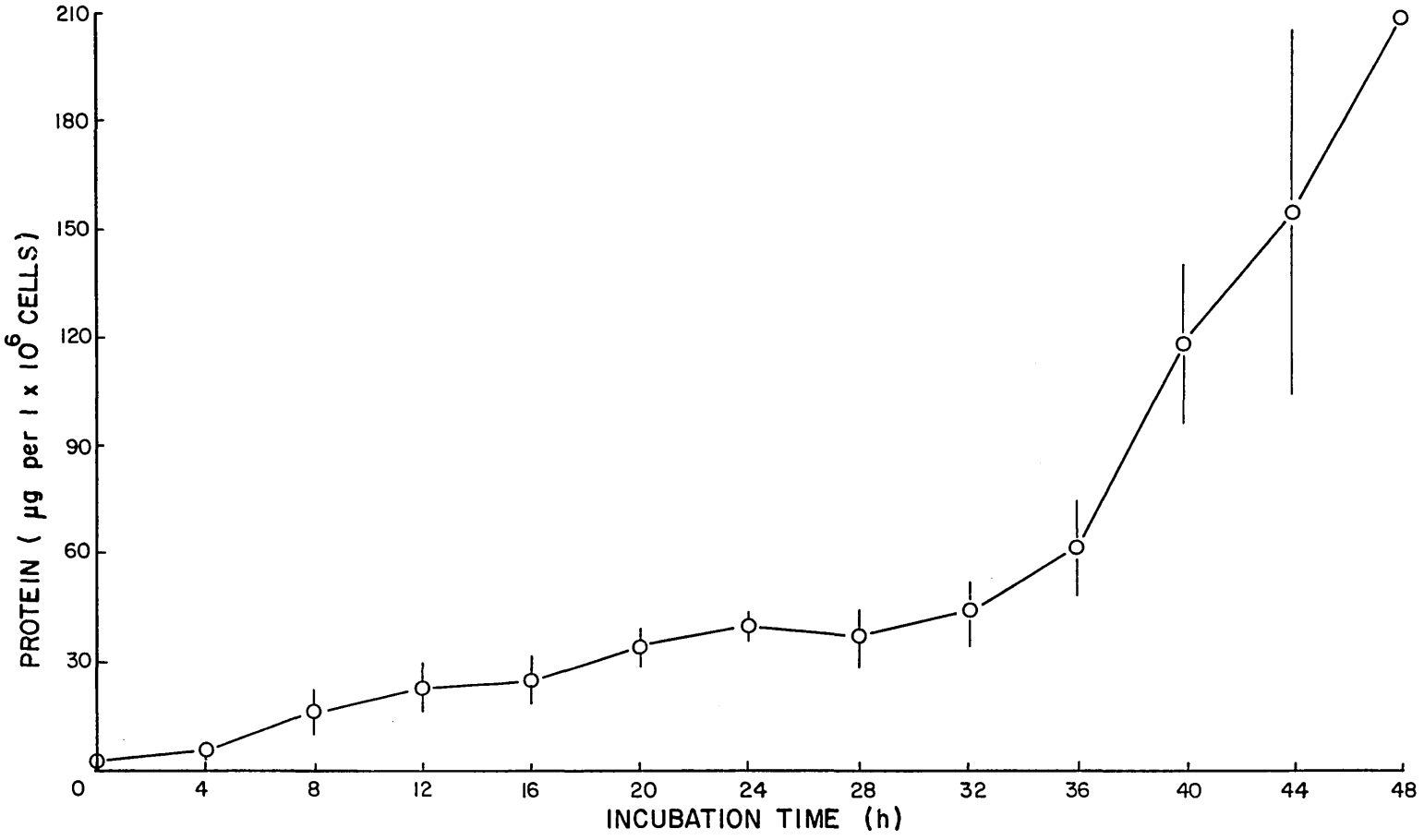


FIG. 4.4 PROTEIN DETERMINATION ON A.NIGER CELLS UNDERGOING MICROCYCLE CONIDIATION IN FERMENTER CULTIVATION.

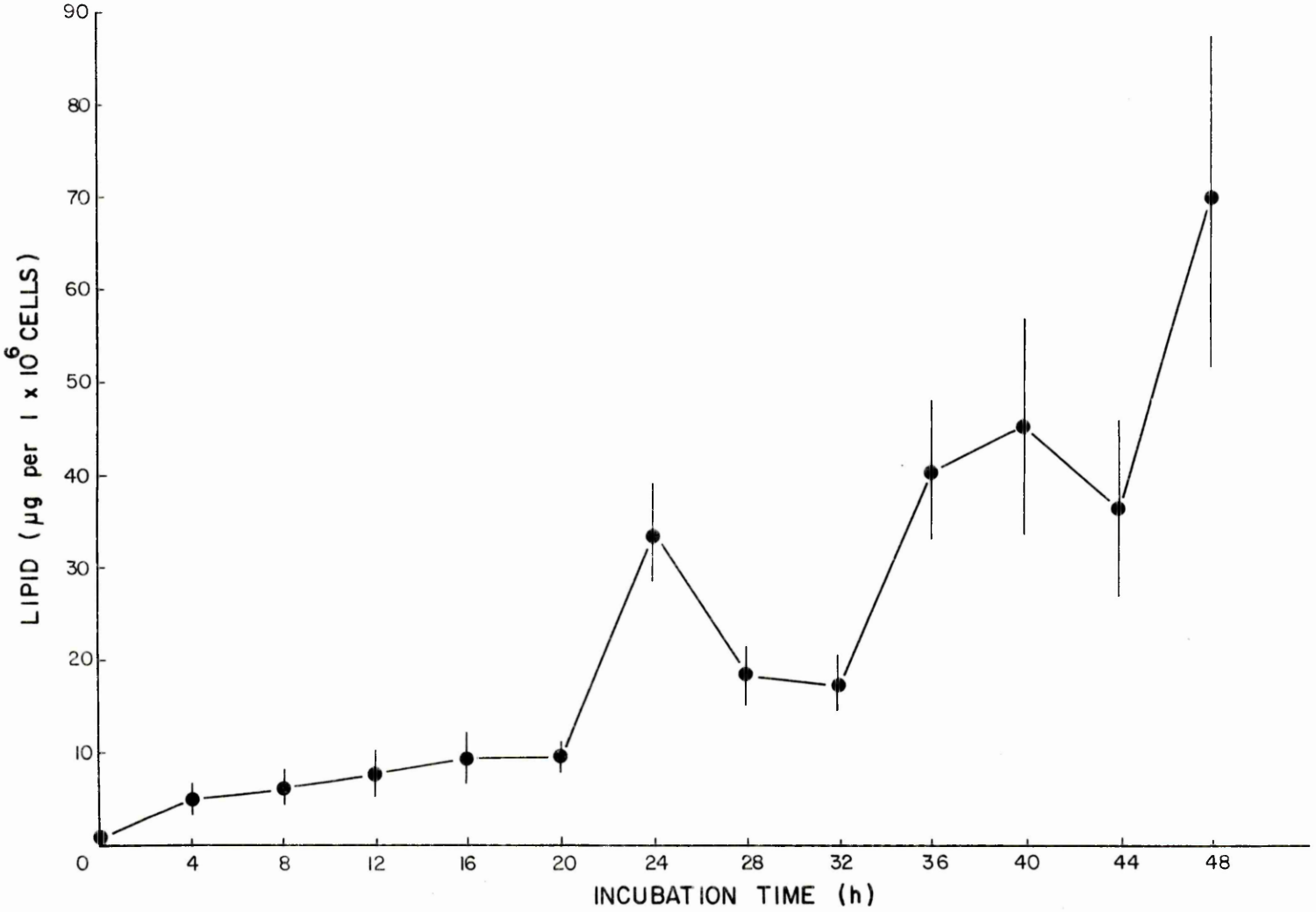


FIG.4.5 TOTAL LIPID CONTENT OF A.NIGER UNDERGOING MICROCYCLE CONIDIATION IN FERMENTER CULTIVATION.

This increase is statistically significant at the 1% level ($P<0.01$).

A decrease in lipid content was noted in the 24 to 28h period of conidiophore initiation. This decrease from 33.9 µg to 18.1 µg of lipid per 1 x 10^6 cells was found to be statistically significant at the 5% level ($P<0.05$). A slight decrease in cellular lipid levels occurred from 28 to 32h followed by a rapid accumulation to 40.6 µg per 1 x 10^6 cells at 36h. There was a slight increase in lipid content to 40h, with a subsequent decrease to 36.2 µg of lipid per 1 x 10^6 cells at 44h. The final four hour period of the microcycle was one of rapid lipid accumulation resulting in lipid levels of 69.7 µg per 1 x 10^6 cells at 48h.

Fig. 4.6 illustrates lipid content during fermenter growth expressed as a percentage of dry weight. This representation shows accumulation of lipid above levels parallel to the increase in dry weight. Thus there is a considerable accumulation at 24h, followed by a decline from 28 to 32h. From 32h there is a second accumulation which however is less rapid than the corresponding total increase in dry weight, as can be seen from a comparison of Figs. 4.3 and 4.6. The third increase shown on Fig. 4.5 is not evident on Fig. 4.6, suggesting that the increase in lipid from 44h to the end of the microcycle is a result of the great increase in biomass, rather than of lipid accumulation *per se*.

4.2.4 Neutral and Polar Lipid Content

The results given in Fig. 4.7 show the changes in the polar and neutral lipid fractions of the total lipid throughout the 48h fermenter microcycle.

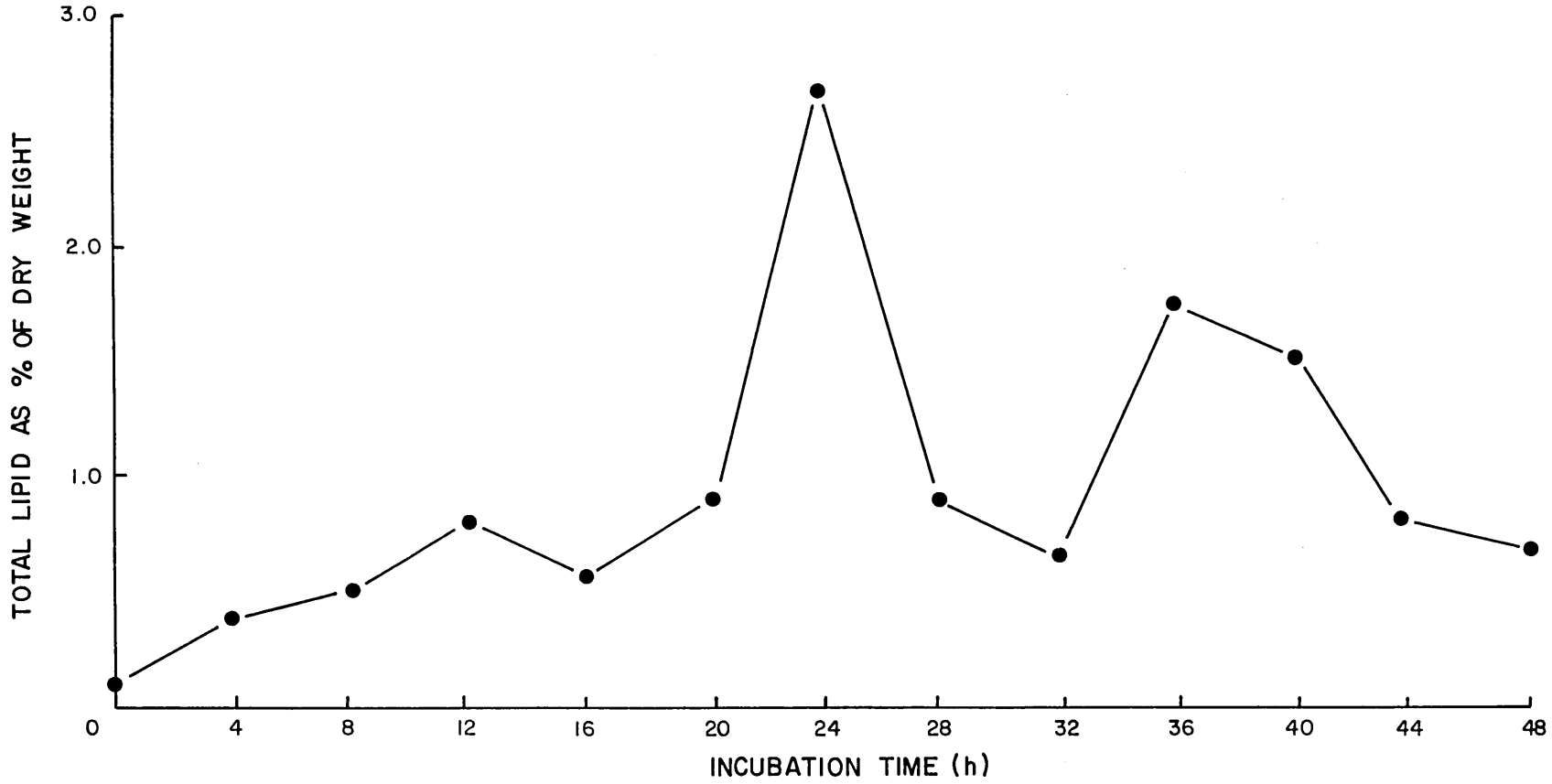


FIG. 4.6 TOTAL LIPID CONTENT OVER THE 48h A. NIGER MICROCYCLE IN FERMENTER CULTIVATION EXPRESSED AS A PERCENTAGE OF CELLULAR DRY WEIGHT.

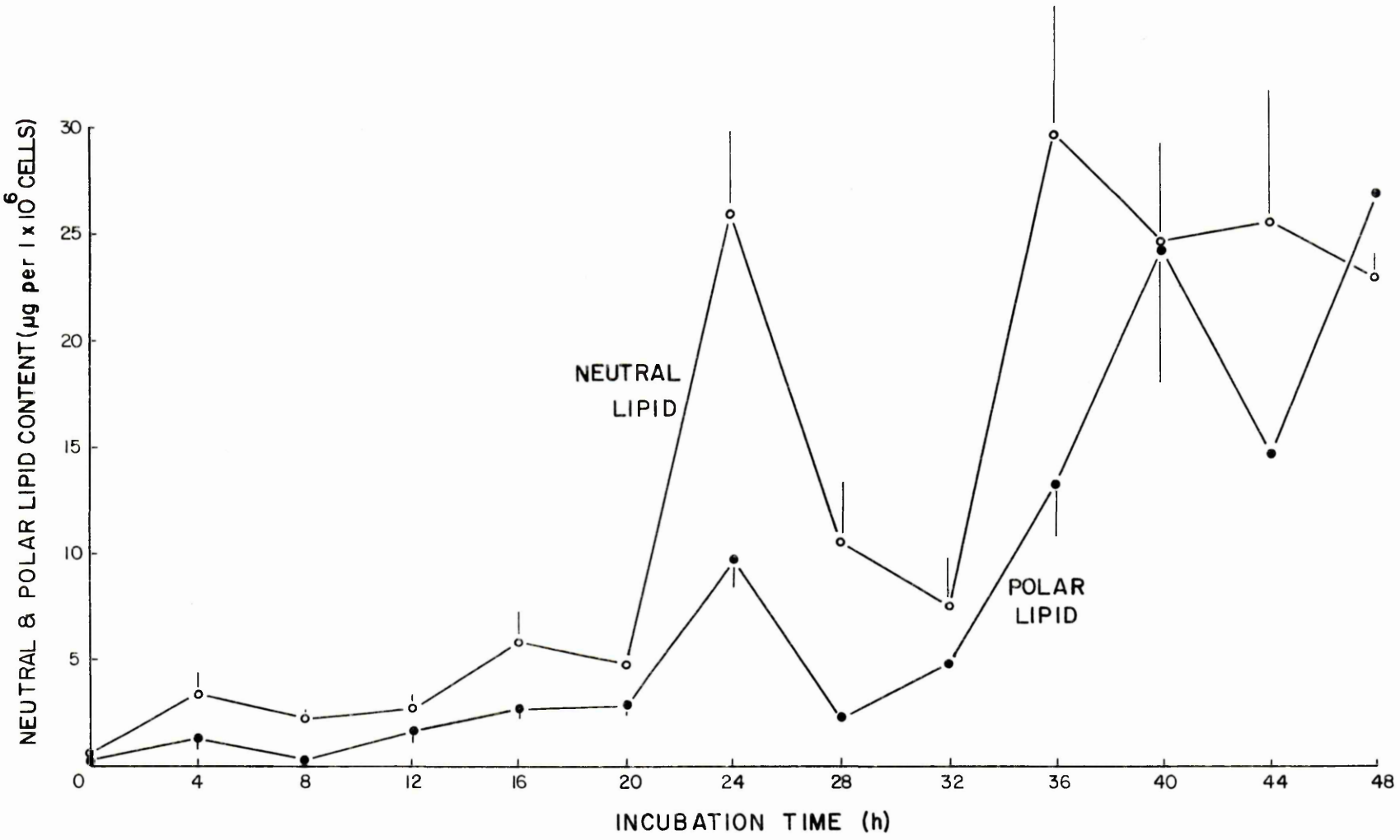


FIG.4.7 LIPID CONTENT OF POLAR & NEUTRAL LIPID FRACTIONS EXTRACTED FROM A. NIGER CELLS UNDERGOING MICROCYCLE CONIDIATION.

They show two peaks of accumulation of neutral lipid at 24h and 36h separated by a large decrease in neutral lipid levels. There was a similar but smaller increase in polar lipid at 24h and a second accumulation of polar lipid at 40h. At 48h the polar lipid content exceeded that of neutral lipid.

The data suggest that the small decrease at 44h in total lipid content may be due to a fall in the polar rather than the neutral lipid level. When the neutral and polar lipids are expressed as a percentage of the total cellular dry weight (Fig. 4.8) then both show a similar profile to the total lipid profile (Fig. 4.5). However, the increase in amount of neutral lipid at 24h is greater in absolute terms than that of the polar lipids. Additionally the decrease observed in neutral lipid after 36h when the data is expressed as the percentage of dry weight is seen to precede the fall in polar lipid content.

4.2.5 Individual Components of the Neutral Lipid Fractions

The neutral lipid fraction was separated into its constituent classes by thin layer chromatography and the components quantified using densitometry as described in Chapter 2.9.2. The major components were sterol ester, triacylglycerol, fatty acid and an additional lipid (Unidentified 11 in Table 4.3) which, on the basis of its tlc mobility, may be monoacylglycerol. Other unidentified components contributed a very low percentage to the total lipid fraction (Table 4.3). The data shown in Fig. 4.9 was used in conjunction with the total neutral lipid data (Fig. 4.7) to calculate the actual levels of the above components throughout the microcycle (Fig. 4.10).

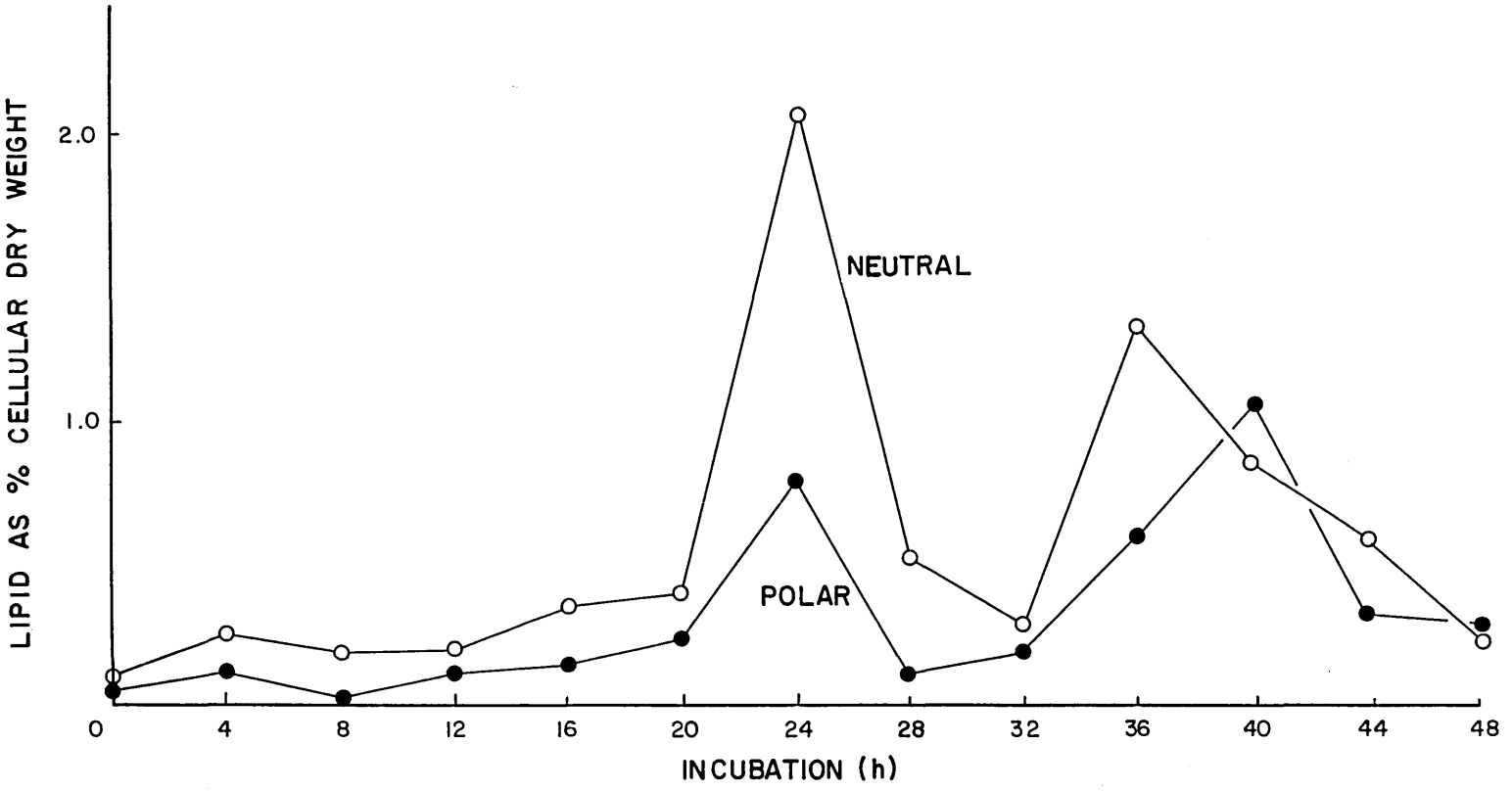


FIG.4.8 NEUTRAL & POLAR LIPID FRACTIONS SEPARATED BY COLUMN CHROMATOGRAPHY & EXPRESSED AS A PERCENTAGE OF CELLULAR DRY WEIGHT.

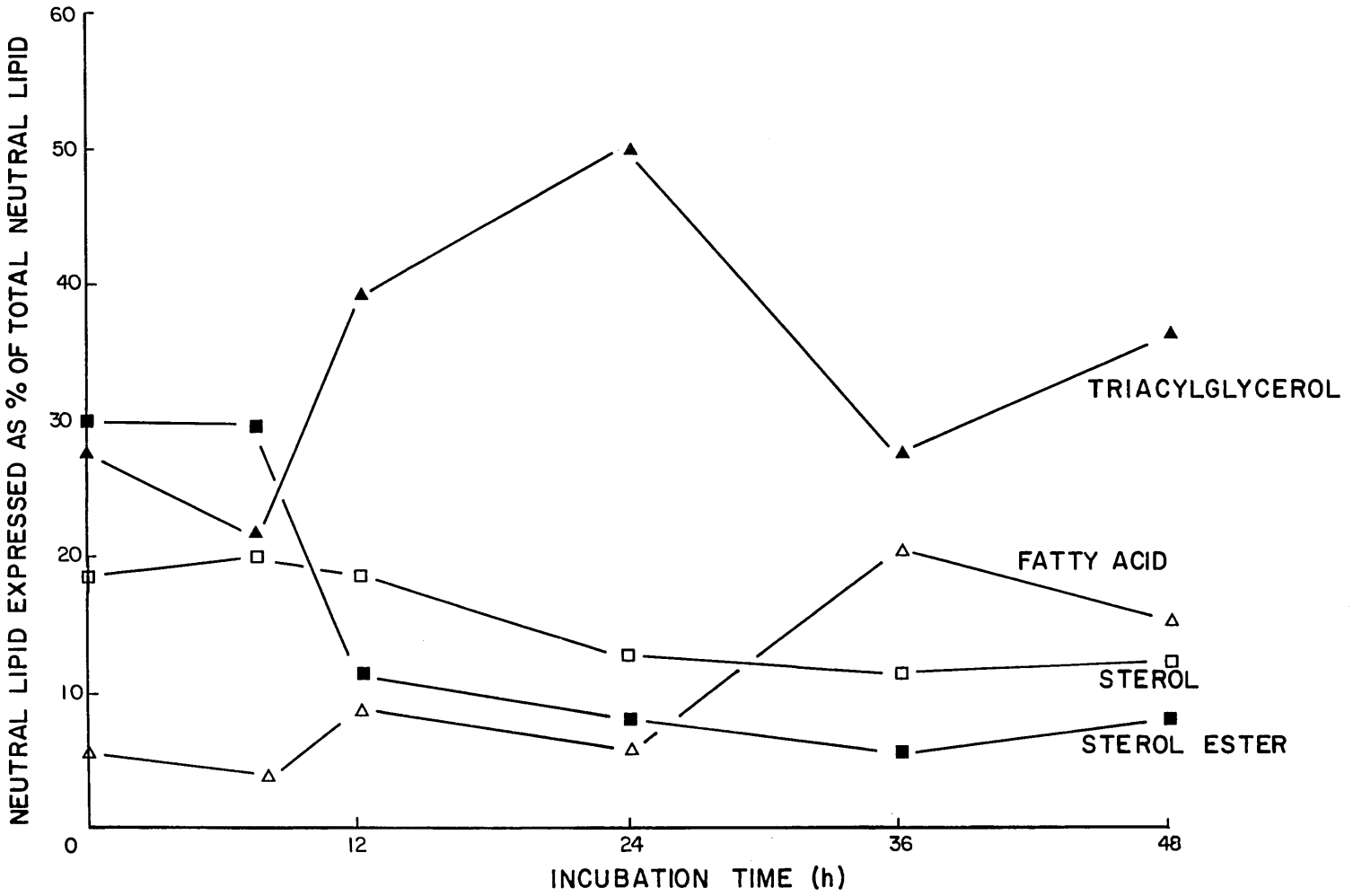


FIG.4.9 THE MAIN COMPONENTS OF THE NEUTRAL LIPID FRACTION EXTRACTED FROM A.NIGER CELLS DURING MICROCYCLE CONIDIATION.

(i) Sterol esters

At the conidial stage, sterol ester is a major percentage constituent of the total neutral lipid present. This relatively high percentage contribution is maintained during the first 8h of giant cell formation, but from 8 to 12h there was a rapid decline in the sterol ester percentage of the neutral lipid with the actual amount decreasing by half (Fig. 4.10). This decline continued steadily in percentage terms to 36h, but the actual amount (µg per 1 x 10^6 cells) increased rapidly from 12 to 24h, with a decrease to 36h. There was a slight increase during the final stages of the microcycle to 48h on both a percentage and weight basis.

(ii) Sterols

The sterol component of the neutral lipid represented 18.5% of the total lipid level in the conidia, rising slightly to 20.1% at 8h and returning to 18.5% at 12h. Although its percentage contribution to total neutral lipid declined from 12 to 36h, the amount of sterol increased dramatically from 12h to 24h, the final period of giant cell formation. The amount of sterol present remained almost constant from 24 to 36h, the period of conidiophore initiation and development. However, during the final twelve hour period of the microcycle there was a slight relative increase in sterol levels from 11.3 to 12.1%, which in weight terms was however, a decrease from 3.37 to 2.81 µg per 1 x 10^6 cells.

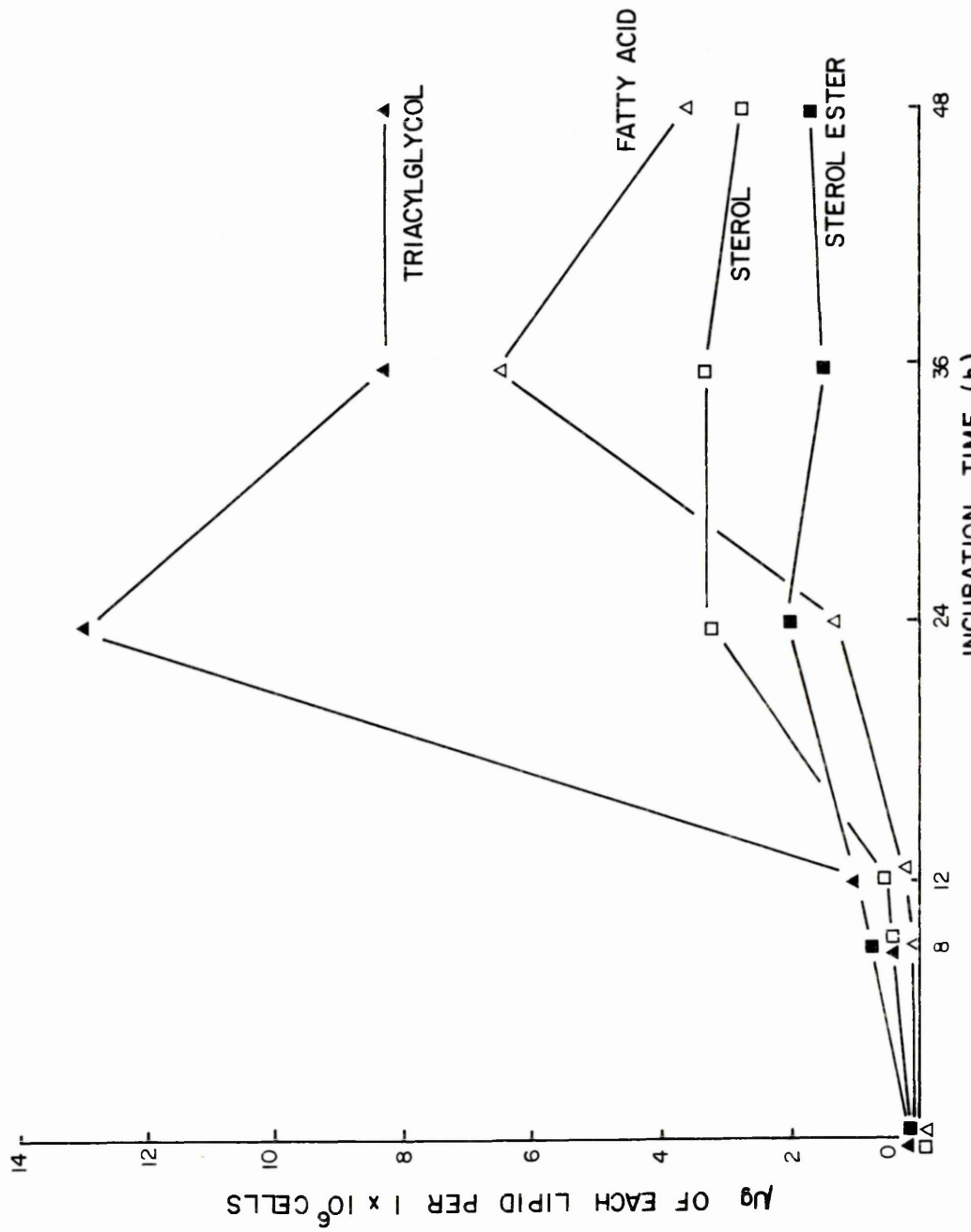


FIG. 4.10 THE AMOUNT OF EACH LIPID CLASS PRESENT THROUGHOUT MICROCYCLE CONIDIATION IN *A.NIGER*.

(iii) Triacylglycerols

Triacylglycerol is a major component of conidial neutral lipid at levels of 27.4%. This relative percentage decreased over the first eight hours of giant cell formation, but this presentation of the data masks triacylglycerol synthesis, as levels rose from 0.09 to 0.46 μg per 1 x 10^6 cells. The triacylglycerol level increased in percentage and real values over the subsequent four hours to 12h. After 12h, triacylglycerol is the major lipid component reaching a maximum of 50% at 24h which corresponds to a peak in total and neutral lipid content in the giant cells (Figs. 4.5 and 4.7). A rapid accumulation of triacylglycerol was noted during the final stages of giant cell formation (12 - 24h) with levels increasing almost thirteen-fold. The triacylglycerol levels fell at 36h, accompanied by an increase in the non-esterified fatty acid content. Thereafter the triacylglycerol percentage level rose with a corresponding slight decline in the non-esterified fatty acid present. The values on a weight basis indicate little change in triacylglycerol levels but a rapid decline in fatty acid values.

(iv) Fatty Acids

The non-esterified fatty acids are at a relatively low level (5.4%) in the conidia. This percentage decreased over the first 8h, rose to 12h and decreased slightly over the latter twelve hour period of giant cell formation to 24h. However, on a weight basis, the fatty acid levels increase steadily to 24h, with a rapid accumulation to 36h.

	Lipid as % total neutral lipid					
Incubation time (h)	0	8	12	24	36	48
Unidentified 1					2.4	3.4
Sterol ester	30.0	29.4	11.1	7.8	5.2	7.8
Unidentified 2	0.7	8.1	0.5			
Unidentifed 3	2.0		2.3		7.5	2.0
Unidentifed 4				2.3		
Triacglglycerol	27.4	21.2	39.4	50.3	27.9	36.5
Unidentifed 5	9.7	2.6		0.8	1.55	
Unidentifed 6						3.5
Unidentifed 7				0.6		
Fatty acid	5.4	3.6	8.3	5.1	21.8	15.7
Unidentified 8						0.5
Unidentified 9			1.1	0.8	1.55	1.6
Sterol	18.5	20.1	18.5	12.6	11.3	12.1
Unidentified 10			1.3			0.2
Unidentified 11	4.3	11.5	9.0	19.3	13.9	8.1
Unidentified 12				0.1		
Unidentified 13				0.1		
Unidentified 14	2.3	3.6	8.3	0.5	7.0	8.9

Table 4.3 Neutral lipids of A. niger undergoing microcycle conidiation expressed as a percentage of total lipid, following separation by tlc.

A high relative value of 21.8% was reached at 36h, declining to 15.7% on completion of the microcycle at 48h. A corresponding high fatty acid level was recorded on a weight basis at 36h, declining rapidly to 48h.

4.2.6 Polar Lipids

Preliminary experiments were carried out on individual polar lipids. Phosphatidyl choline and phosphatidyl ethanolamine were noted as two of the major components of the polar lipid fraction with a number of more minor constituents present. Visual interpretation of the tlc separated polar lipids suggest that the pattern of polar lipid component varies over the 48h period of microcycle conidiation. Further work would be required to confirm these observations.

4.2.7 Fatty Acid Composition of the Giant Cells

The proportion of various fatty acids present in the lipids extracted from giant cells at times throughout the microcycle are shown in Figs. 4.11, 4.12, 4.13 and 4.14. Figs. 4.11 and 4.12 illustrate the fatty acid content of the total lipid extraction whereas Figs. 4.13 and 4.14 show the fatty acids present in the triacylglycerol fraction which was isolated by tlc. The major fatty acids in both cases were palmitic, stearic, oleic, linoleic and linolenic acids. These accounted for at least 80% of the total fatty acid content of the triacylglycerol fraction and over 96% of the total lipid fraction.

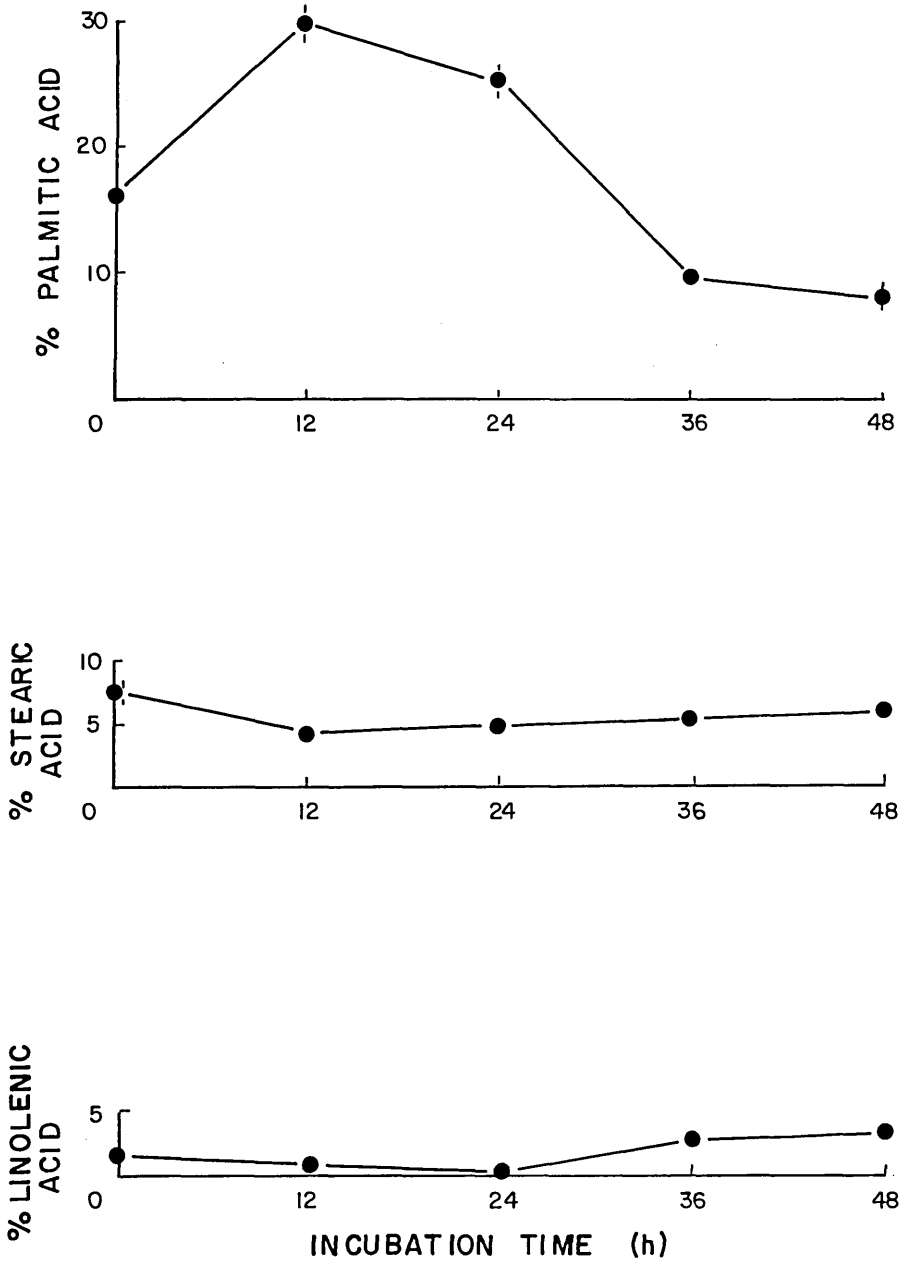


FIG.4.11 PALMITIC, STEARIC AND LINOLENIC ACID CONTENT OF A.NIGER CELLS THROUGHOUT MICRO-CYCLE CONIDIATION, EXPRESSED AS A PERCENTAGE OF THE TOTAL FATTY ACID PRESENT.

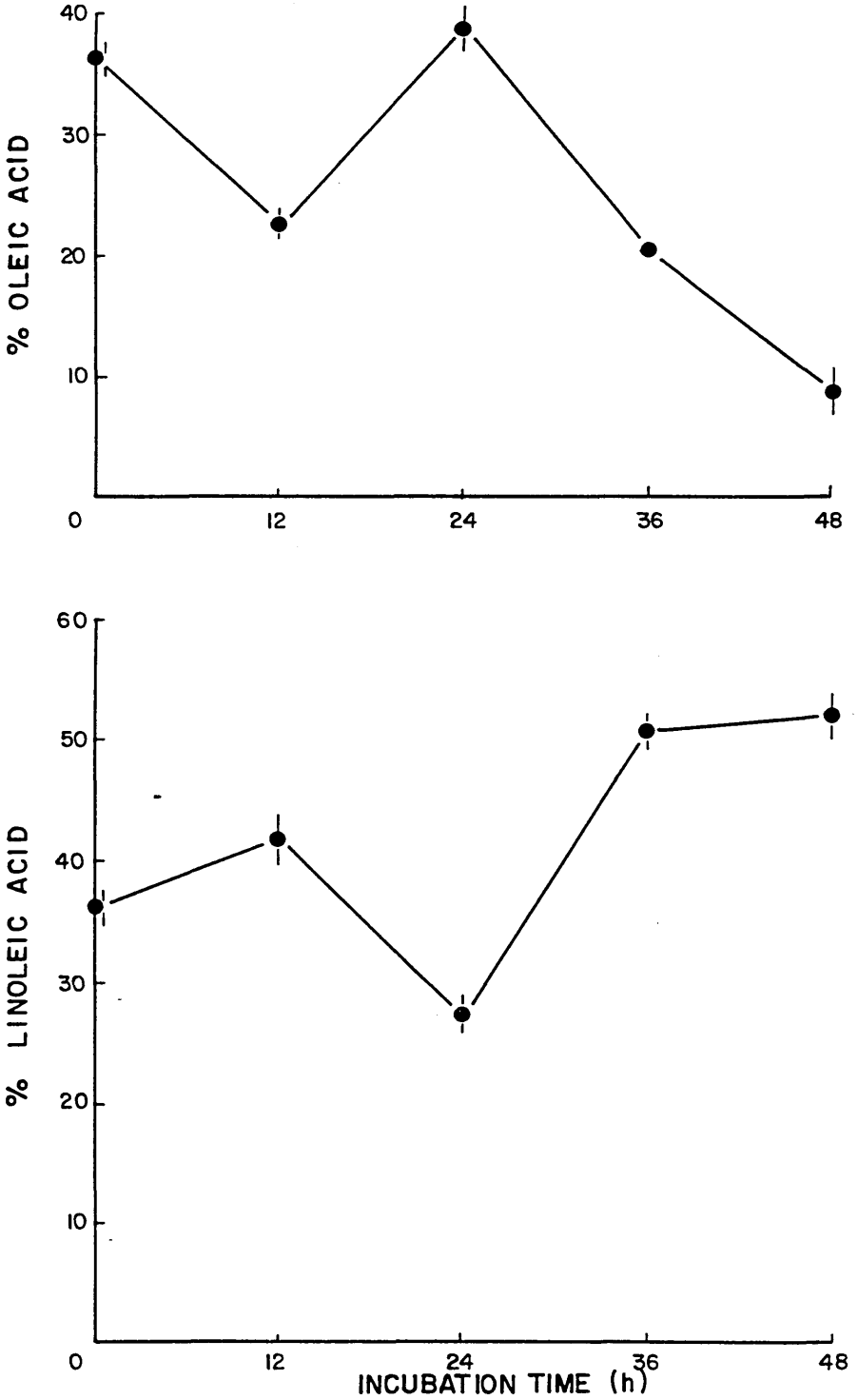


FIG.4.12 OLEIC AND LINOLEIC ACID CONTENT OF A. NIGER CELLS THROUGHOUT MICROCYCLE CONIDIA-TION, EXPRESSED AS A PERCENTAGE OF THE TOTAL FATTY ACID PRESENT.

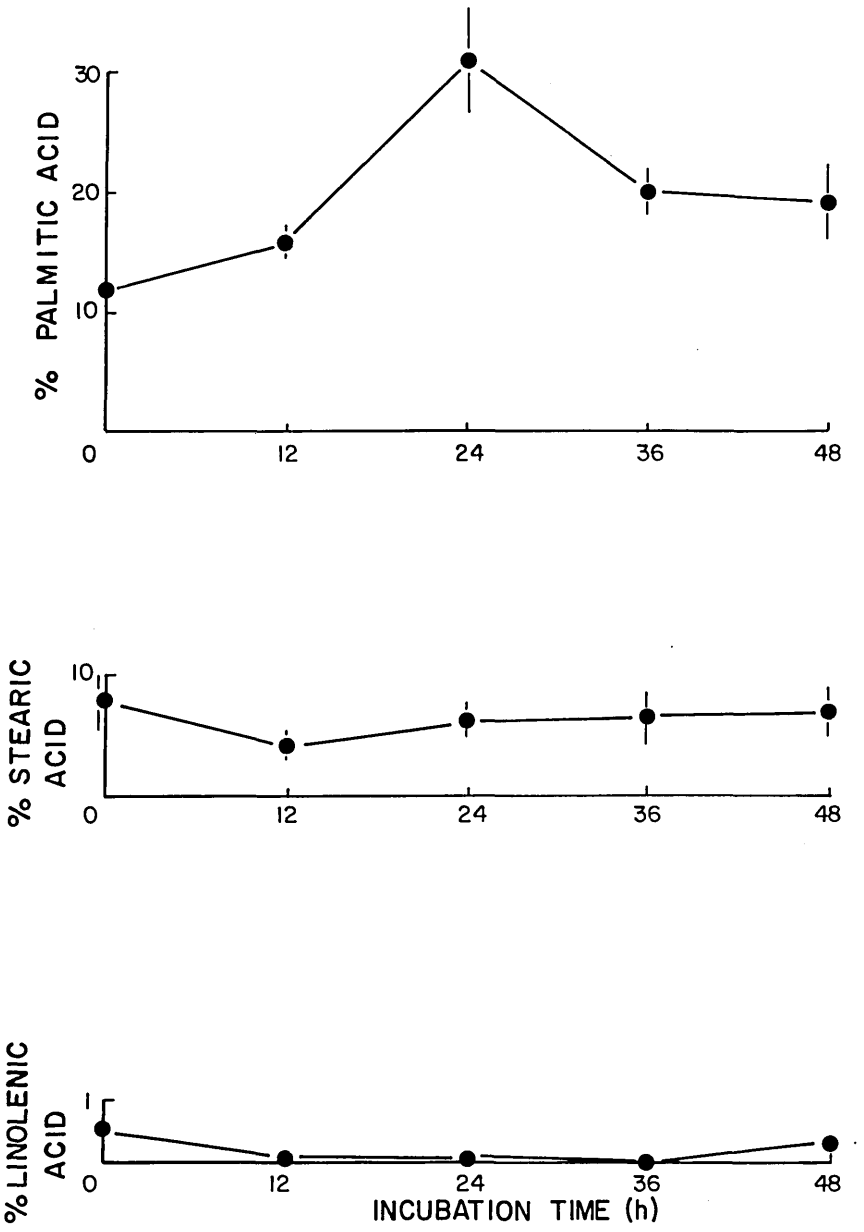


FIG.4.13 PALMITIC, STEARIC AND LINOLENIC ACID IN A. NIGER CELLS THROUGHOUT MICROCYCLE CONIDIATION, EXPRESSED AS A PERCENTAGE OF THE FATTY ACIDS PRESENT IN THE TRIACYLGLYCEROL FRACTION OF LIPID EXTRACTION.

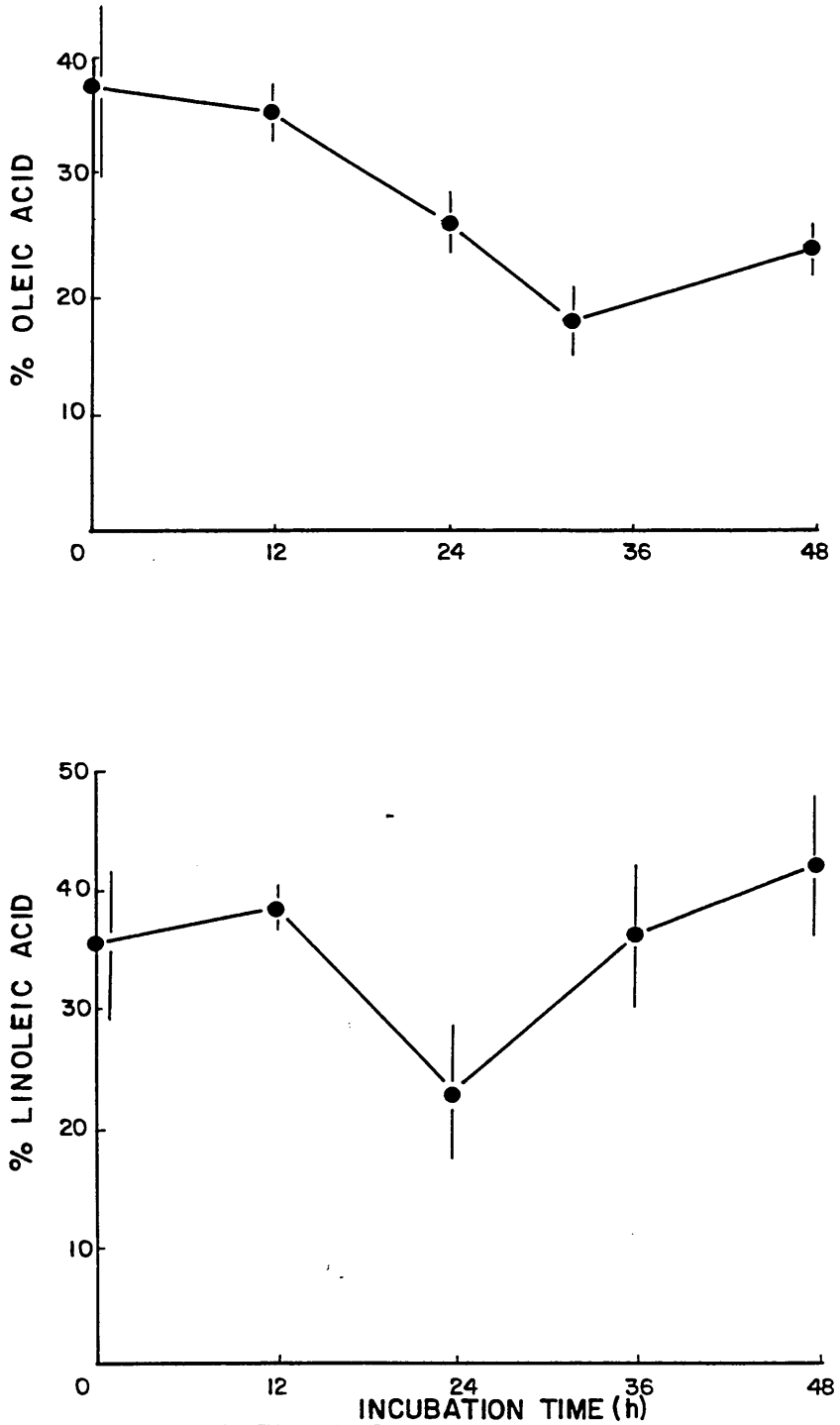


FIG.4.14 OLEIC AND LINOLEIC ACID CONTENT OF A. NIGER CELLS THROUGHOUT MICROCYCLE CONID--IATION, EXPRESSED AS A PERCENTAGE OF THE FATTY ACIDS PRESENT IN THE TRIACYLGLYCEROL FRACTION OF LIPID EXTRACTION.

Other individual components accounted for no more than 1.7% of the total fatty acid at any one time. Of the major fatty acids present, linoleic acid was the most abundant reaching a maximum of 52.0% at 48h in the total lipid analysis. Oleic acid was at its maximum of 37.6% of the total fatty acid in the triacylglycerol fraction of conidial lipid, with palmitic acid reaching a maximum of 30.6% at 24h in this fraction. Stearic acid was present at lower percentage levels with a maximum of 7.9% in conidial lipid. Linolenic acid was present at low levels ranging from zero to 3.0% of the total fatty acid content. Whereas the relative percentage values of palmitic and linoleic acids increase, the corresponding values for stearic, oleic and linoleic acids decrease. A similar trend in percentage terms was noted for the fatty acids of the triacylglycerol fraction although the changes, particularly in palmitic and oleic acid levels were less pronounced than in the total lipid analysis.

During the second twelve hour period of giant cell formation, the percentage of oleic acid present rose dramatically accompanied by a rapid decline in linoleic acid percentage levels. A slight decrease in the percentage of palmitic and linolenic acids was recorded over the 12 to 24h period with an increase in stearic acid levels. However, in the triacylglycerol fraction, the palmitic acid percentage content increased accompanied by a sharp decline in both oleic and linoleic percentage levels. The stearic acid levels increased in relative terms, with little change in the linolenic acid percentage present.

During the subsequent 24 to 36h period at 30°C, the relative levels of palmitic and oleic acids decreased in both the total lipid and triacylglycerol fractions with a corresponding increase in linoleic percentage content. Little change occurred in stearic acid levels in the total lipid fraction but there was an increase in the linolenic acid percentage. However, in the triacylglycerol fraction the linolenic acid percentage levels decreased with an increase in stearic acid levels.

Little change occurred in the percentage levels of palmitic and stearic acids present in both the total and triacylglycerol fractions over the final twelve hour period of the microcycle to 48h. A sharp decline in the percentage of oleic acid present in the total lipid fraction was noted, although an increase occurred in the relative amount of this fatty acid present in the triacylglycerol fraction. Linoleic acid levels increased in percentage terms in both fractions to reach its maximum value for the microcycle. The linolenic percentage levels, although very low throughout the microcycle, increased during the final twelve hour period.

The trends in total saturated and total unsaturated fatty acid percentage levels over the 48h microcycle are shown in Figs. 4.15 and 4.16. Fig. 4.15 illustrates the trends in fatty acid saturation in the total lipid fraction, Fig. 4.16 shows the levels in the triacylglycerol fraction. There was an increase in the percentage of saturated fatty acids accompanied by a corresponding decrease in unsaturated fatty acid levels in the total lipid extracted from the microcycle cells during the 0 to 12h period. However, little change occurred in the degree of saturation of the fatty acids in the triacylglycerol fraction over this first twelve hour period.

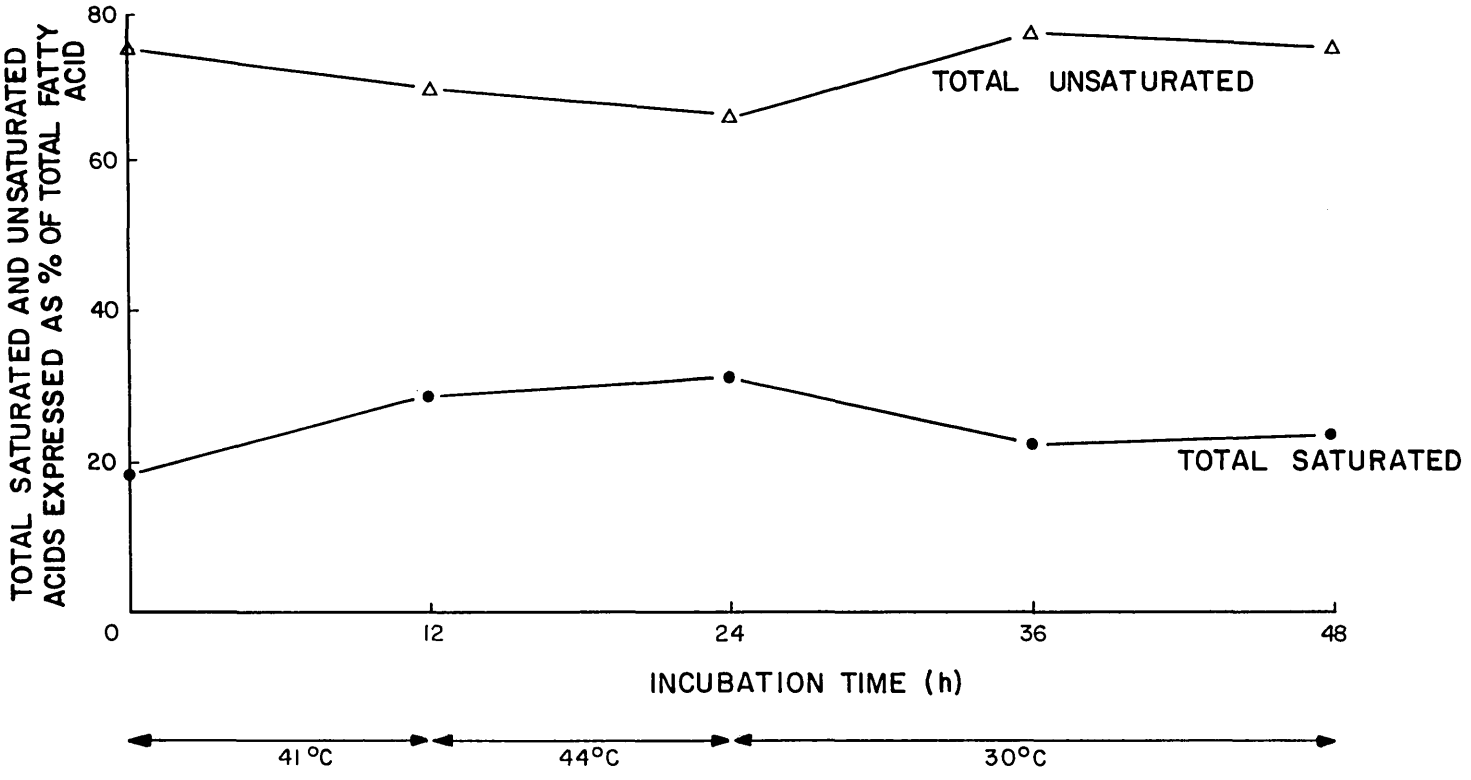


FIG. 4.15 TOTAL SATURATED AND TOTAL UNSATURATED FATTY ACIDS FOUND IN A. NIGER DURING MICROCYCLE CONIDIATION EXPRESSED AS A PERCENTAGE OF TOTAL FATTY ACID CONTENT.

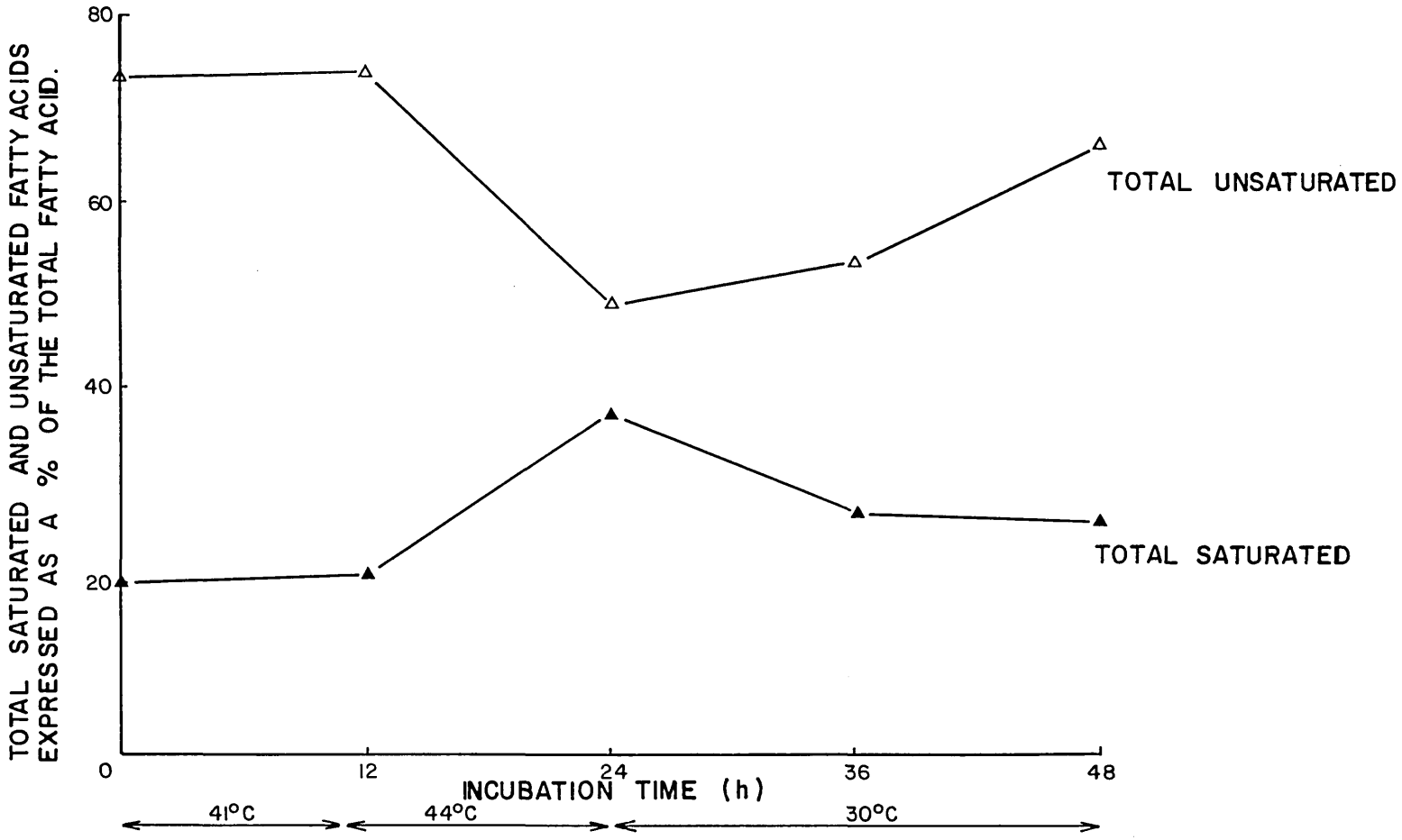


FIG.4.16 TOTAL SATURATED AND TOTAL UNSATURATED FATTY ACIDS IN THE TRIACYLGLYCEROL FRACTION OF THE LIPID EXTRACTED FROM A.NIGER GIANT CELLS EXPRESSED AS A PERCENTAGE OF THE TOTAL FATTY ACID CONTENT.

During the subsequent 12 to 24h period at 44°C there was a marked trend towards saturation of the fatty acids in the triacylglycerol fraction with an overall slight increase in saturation noted for the total lipid fraction. Following the decrease in temperature to 30°C at 24h, decreased saturation of the fatty acids was noted in both fractions. The ratio of saturated to unsaturated fatty acids remained almost constant over the final twelve hour period of the microcycle in the total lipid fraction but greater unsaturation was noted in the triacylglycerol fraction. The degree of fatty acid unsaturation over the microcycle is given in Table 4.4. It is apparent that the highest degree of fatty acid unsaturation occurred in the total lipid extract from cells growing at the lower temperature of 30°C at 36 and 48h. The lowest degree of unsaturation was to be found in both the total lipid and triacylglycerol fractions at 24h when the cells had been growing at the higher temperature of 44°C.

Incubation time (h)	Degree of unsaturation per mole	
	Total fatty acid	Triacylglycerol fatty acid
0	1.13	1.10
12	1.08	1.12
24	0.94	0.73
36	1.30	0.89
48	1.32	1.08

Table 4.4 The degree of fatty acid unsaturation in A. niger cells during microcycle conidiation.

The degree of fatty acid unsaturation per mole was calculated from the data in Figs. 4.15 and 4.16 using the following formula (see Weete, 1980):-

Degree of fatty acid unsaturation per mole (Δ/mole)

$$= \frac{(1 \times \% \text{ Monoenes})}{100} + \frac{(2 \times \% \text{ dienes})}{100} + \frac{(3 \times \% \text{ trienes})}{100}.$$

4.3 DISCUSSION

4.3.1 Spore lipid and its role in germination

The A. niger conidial lipid content is 0.11% of the cellular dry weight in the present study. This is lower than that reported for other fungal spores of various species which have a lipid content of 1-35% (see Chapter 1). The low lipid content of A. niger conidia could suggest that although lipid may be essential for spore germination it is not a major reserve material.

The total amount of lipid accumulating during microcycle growth of A. niger is much less than that reported for many filamentous fungal mycelia. The total lipid of vegetative hyphae varies greatly between species and is affected by environmental conditions. A lipid content of 3-32% of the cellular dry weight is reported for most fungi cultured under conditions favourable for growth which are not necessarily the optimal conditions for lipid production (see Weete, 1980). Fungi generally average about 17% lipid with Aspergillus clavatus containing 14.2-20% lipid (Woodbine et al., 1951; Murray et al., 1953); various Humicola species 8.0-17.2% (Mumma et al., 1970); Penicillium chrysogenum, 1.2-9.8% (Gaby et al., 1957; Mumma et al., 1970) and various Mucor species, 7.8-21.2% (Sumner and Morgan, 1969; Sumner et al., 1969).

The total lipid content of A. niger vegetative mycelial growth has been reported as 0.9-2.2% of the cellular dry weight (Woodbine et al., 1951) and as 9.8% of the biomass estimated as dry weight by Chahal et al. (1979).

This compares with the fully developed vegetative state of the swollen giant cell at 24h which has a lipid content of 2.7% of the dry weight. The cell wall lipid content of A. niger undergoing microcycle conidiation has been reported as 3.5% of the wall fraction on a dry weight basis (Deans, 1978). The level of cell wall lipids decreased during the first 12h of giant cell formation, then increased gradually to 24h. Following the lowering of the temperature, cell wall lipids accumulated to a maximum, six hours into the conidiophore development stage (Deans, 1978), equivalent to 30h in the present study. There was a decrease during the subsequent 12h and an increase towards the end of the cycle coinciding with conidia formation. Deans' (1978) microcycle conidiation was achieved using a temperature regime of 44^{o}C for 24h, followed by 24h at 30^{o}C. These conditions were the same in the present shake tube culture experiments the results from which are illustrated in Fig. 4.17. The total lipid levels in the cells increased steadily up to 6h into conidiophore production when the phialides would be partially developed. A further more rapid accumulation of lipid occurred in the subsequent 8h during which time the phialides will have become fully developed and conidia will have been produced in the more rapid microcycle of the present study. The next 4h period shows a slight decline in lipid levels corresponding to the final stages of conidial production, and during the subsequent 4h period of conidia release there is a more marked decrease in cellular lipid (Fig. 4.17). It seems that although some cellular lipids including those in the cell wall may be used as substrates during the microcycle, there is an overall accumulation of lipid to 42h in shake tube incubations.

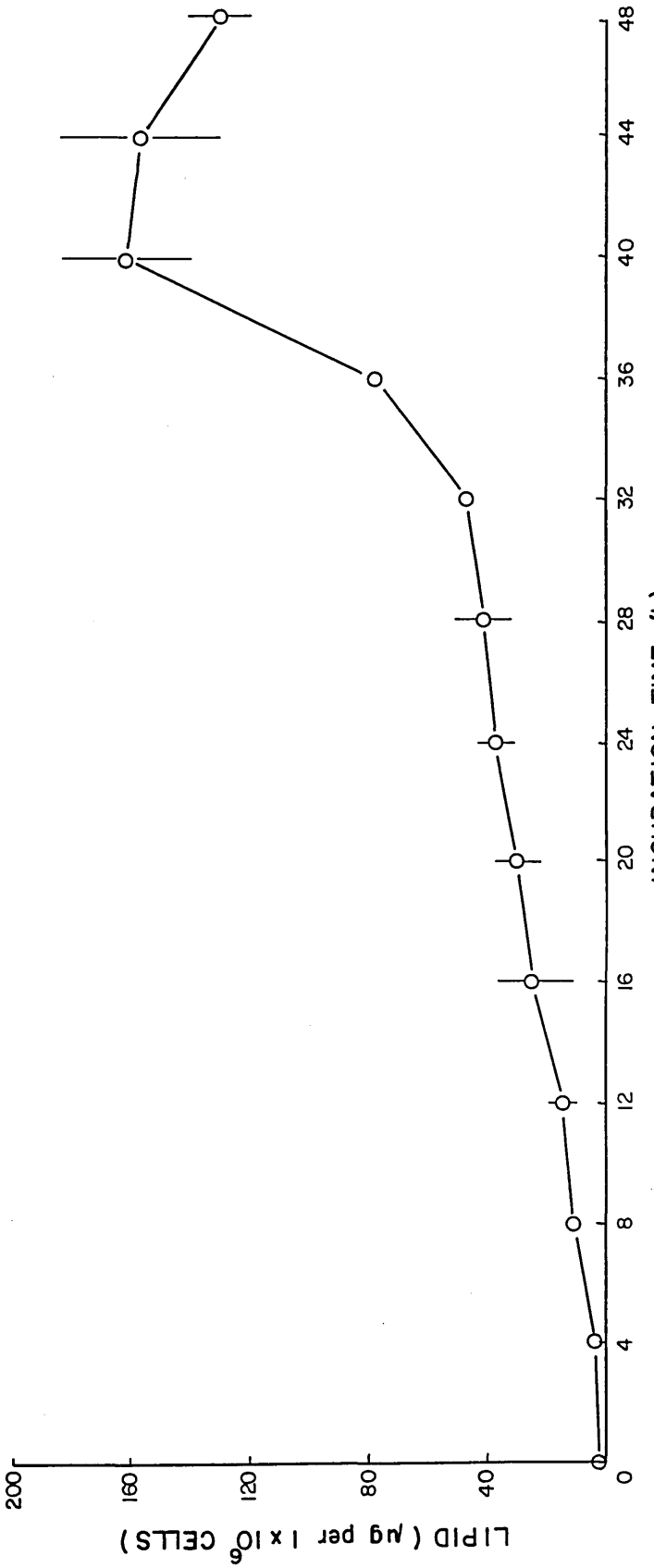


FIG. 4.17 TOTAL LIPID CONTENT OF A.NIGER CELLS DURING MICROCYCLE CONIDIATION IN SHAKE TUBE CULTURE.

The decrease in lipid during the final stages of the microcycle can be attributed to their use as substrates as the medium glucose supply is depleted.

Total accumulation of lipid during the microcycle in fermenter culture showed a gradual increase over the first 16h, followed by a decrease to 20h, with a subsequent rapid increase in lipid levels to 24h (Fig. 4.5). This first 24h phase represents the period of spherical growth from spore to giant cell in which the cell diameter is increased to approximately four times its original size. During this period there is also an increase in lipid expressed as a percentage of cellular dry weight except for a small decrease at 16h (Fig. 4.6). Thus during most of the giant cell formation period, lipid is accumulated in excess of the increase in biomass. The slight decline in cellular lipid levels from 16 - 20h and the decline at 16h when expressed in dry weight terms may be explained by the demand for substrates required for the rapid spherical growth of the cells being greater than the capacity of the transport system of the cell membranes to supply external nutrients. Hence an internal reserve material such as lipid may be utilised in metabolism. The surface area to volume ratio of the giant cell at 16h is greatly reduced, resulting in a decrease in the relative cell membrane area through which external nutrients enter the cell. From a comparison of the neutral and polar lipids (Fig. 4.7) in the giant cell, it is apparent that the polar fraction increases steadily from 12 - 20h. However there is a decrease in neutral lipid content from 16 - 20h which could be attributed to the use of triacylglycerols as an energy source.

The neutral and polar lipid content of A. niger mycelial growth has been reported as 66.8% and 33.2% of total lipid respectively (Chahal et al., 1979) which compares with values at 24h in microcycle of 72.5% and 27.5%.

After 24h of spherical growth, the temperature was decreased to 30°C which resulted in conidiophore emergence with conidiation complete at 36 - 44h. In this second 24h period, the dry weight and protein content continued to show a general increase (Figs. 4.3 and 4.4), with a rapid increase after 36h. There was a decline in lipid content in the 24 - 32h period in contrast to the increase in dry weight. Deans (1978) also showed a similar decline in cell wall lipid over this period. This suggests a role for lipids as metabolic substrates for the initiation of conidiophore production and in the early stages of its development. Although the triacylglycerols are usually regarded as the lipid class used as energy reserves, the decrease in polar lipid (Fig. 4.7) and cell wall lipids (Deans, 1978) may indicate a role for phospholipids as a substrate for energy metabolism or as carbon skeletons for the developing conidiophore structures. Over the initial period of conidiophore emergence, which is an energy demanding process, the medium glucose declined only slightly. This tends to confirm the importance of internal reserve materials at this stage. From 36h to 40h, the glucose and amino nitrogen levels declined rapidly (Figs. 6.4 and 6.5) suggesting their utilisation during the later stages of conidiophore production. At this time lipid accumulated but no longer kept pace with the increase in biomass estimated as dry weight.

Although A. niger cell wall lipids declined rapidly during the first 8h of giant cell formation (Deans, 1978) the polar lipid content of the whole cell increased during the first 4h, and then decreased to 8h. A similar pattern is followed by the neutral lipid content suggesting that either external nutrients or other forms of storage materials are more important during the initial stages of spore germination, with both neutral and polar lipids utilised in the 4 - 8h phase.

There is a rapid accumulation of both polar and neutral lipids during the last 4h of giant cell formation with a particularly marked increase in the neutral fraction. Deans (1978) also reported a rapid increase in cell wall lipids during the final stages of giant cell growth. The neutral lipids and to a lesser extent, the polar lipids represent a convenient storage system, although the latter may function in the elaboration of the main cell wall polysaccharides (see Smith et al., 1981a;b). Conidiophore emergence is accompanied by a rapid decline in both polar and neutral lipid content suggesting a role for both fractions as energy sources and/or carbon skeletons. However, conidiophore elaboration with the production of phialides and conidia is accompanied by a rapid increase in both neutral and polar lipid content presumably due to their synthesis in the development of these new structures. Deans (1978) also reported a drop in cell wall lipid content during conidiophore development, but an increase during conidial production. In the present study, the neutral lipid declined in the 36 - 40h period perhaps due to its utilisation in the production of new conidia.

Although both neutral and polar lipids are accumulated in the mature conidiophores, the amounts synthesised are outpaced by the increase in biomass estimated as dry weight. Neutral lipids show a decline during the final 4h of the microcycle and are probably used as a reserve metabolic source as there is almost complete depletion of the main medium carbon source, glucose, at this stage. At 48h, the polar lipid content was greater than the neutral lipid content, suggesting proliferation of cell membranes in new cellular material which is supported by the dramatic increase in dry weight at this time (Fig. 4.3).

4.3.2 Analysis of Lipid Composition

(i) Lipid Classes

The neutral lipids of a number of fungi have been analysed by other workers (Table 4.5). The predominant neutral lipids are sterol esters, triacylglycerols, fatty acids and sterols which are also the main classes reported in the present A. niger microcycle work. These classes together with hydrocarbons have been previously reported in A. niger mycelia (Chahal et al., 1979). The main polar lipids are phospholipids and to a lesser extent glycolipids. The relative proportions of the lipid classes present may vary according to the age of the fungal culture, environmental conditions, and the developmental stage. These factors must be considered when comparing lipid contents. In general the neutral lipids tend to accumulate with the age of the fungus. Triacylglycerols may comprise up to 90% of the lipid present in fungi (see Weete, 1980).

Fungi		% Total neutral lipid			
		Sterol esters	Triacyl glycerols	Fatty acid	Sterol
Cephalosporium falciform	(a)	17.0	47.2	7.5	24.5
C. kiliense	(a)	31.4	35.3	5.9	27.5
Saccharomyces cerevisiae	(b)	32.0	50.0	12.0	6.0
Pithomyces charatum	(c)		75.3	24.6	
Stemphylium dendriticum	(c)		81.3	18.8	
Cylindrocarpon radioicola	(c)		85.5	14.5	
Neurospora crassa	(d)	0.9	87.6	2.1	5.6
N. crassa	(e)	22.4	3.0	22.4	52.2
Smittium culisetae	(f)		38.0	20.1	28.7
Rhizopus arrhizus	(g)		39.7	21.0	30.0
Ustilago bullata	(h)		41.3	11.1	19.8
U. maydis	(h)		22.3	4.5	14.0
Blastocladiella emersonii	(i)	11.9	30.0	8.1	15.2
Mucor rouxii	(j)		57.8	6.4	34.3
Pythium ultimum	(k)		63.0	34.7	
Saccharomyces cerevisiae	(l)	73.2	13.4	2.1	11.3
S. carlsbergensis	(l)	76.7	11.8	2.7	8.8
Candida utilis	(l)	75.9	16.1		8.0
Schwanniomyces occidentalis	(l)	18.2	43.6	5.7	32.5

Table 4.5 Neutral lipid composition of some fungi (from Weete, 1980)

(a) Sawicki & Pisano, 1977; (b) Hunter & Rose, 1972;
(c) Hartman et al., 1962; (d) Kushuaha et al., 1976;
(e) Bianchi and Turian, 1967 (conidia 6h after germination);
(f) Weete, 1980; (g) Weete et al., 1970; (h) Gunasekaran et al., 1972,b; (i) Mills and Cantino, 1974 (Zoospores); (j) Safe & Duncan, 1974; (k) Bowman and Mumma, (1967); (l) Johnson et al. (1972).

N.B. Sterol esters, hydrocarbons and methyl esters of both Ustilago species were reported as a single value.

The relative proportion of triacylglycerol present in the swelling giant cells decreases in percentage terms during the first 8h, of the microcycle although a real increase from 0.088 to 0.456 µg per 1 x 10^6 cells occurred. During the subsequent sixteen hours at higher temperatures there is a relative percentage increase in triacylglycerol levels reaching a peak at 24h at the final stage of giant cell formation. The real values indicate a gradual increase from 8 to 12h, with a rapid accumulation from 12 to 24h. The giant cell may be using triacylglycerol to accumulate reserve material at this stage. The decline during the period of conidiophore production indicates its subsequent utilisation for cellular metabolic processes. The triacylglycerol level in percentage terms decreased at the end of the microcycle although there was no further real accumulation.

The sterol and sterol ester percentage levels in the A. niger conidia are relatively high and are maintained at such levels during the first 8h of giant cell formation. This may indicate a role for sterols in the initiation and the preliminary stages of spore germination. Weete & Laseter, (1974) reported that A. niger conidia contained at least three C_{28} sterols, the most abundant being ergosterol. The second in abundance was a tetraunsaturated sterol and a diunsaturated C_{28} sterol was a minor component. The sterol level of many fungal species increases until a "threshold" needed for sporulation is attained, which itself is accompanied by sterol loss. However, during the microcycle there is little detectable change in sterol levels during conidiophore production. There are changes in free sterol levels before and after microconidial differentiation in Neurospora crassa indicating a change in lipid metabolism associated with differentiation.

There is a synthesis and accumulation of free sterols prior to the transition from vegetative to reproductive growth and the preferential storage of sterols and sterol esters in the microconidia (Ballou & Bianchi, 1978). There are also reports that many fungi require sterols for the production of sexual spores (Hendrix, 1964; 1970; Weete, 1973).

The free fatty acid as a percentage of total lipid is relatively low in the A. niger conidia and decreases slightly during the first 8h of the microcycle although there is an increase in the amount of fatty acid present. The accumulation to 12h could be correlated to the decrease in sterol esters at this stage releasing fatty acids. The increase in fatty acid continued gradually to 24h which might be correlated to the increase in triacylglycerol synthesis. The relative percentage of fatty acid present, however, decreased slightly over this 12 - 24h period. Definite conclusions would need further data than the composition which takes no account of the rate of turnover.

The production of conidiophores is accompanied by an increase in free fatty acid levels which correspond to a decrease in triacylglycerol levels in both weight and percentage terms. This could be due to the cleavage of the ester bonds of triacylglycerols, so releasing fatty acids which would be available for oxidation. Free fatty acid levels fall towards the end of the microcycle indicating they are incorporated into new cellular material in the form of triacylglycerols and phospholipids or alternatively used as an energy source. It must be noted however that fatty acids rarely exist in the free state in nature and where they are reported as major constituents they are usually artefacts due to cell damage which allows lipases to act on acyl lipids.

However, during the microcycle lipid analysis procedure, care was taken to de-activate any lipase present immediately on sampling.

Preliminary work on the chromatographic separation of the lipid classes in the polar lipid fraction indicate the presence of a variety of compounds which appear to vary in relative proportion over different stages of the microcycle. Polar lipids are mainly phospholipids which are essential structural components in all fungal membranes. The polar lipid in fungi accounts for an average of 41% of the total lipid, the proportion in the fully formed giant cell is 27.5%. Acyl exchange between triacylglycerols and phospholipids is well known in many organisms e.g. Neurospora crassa (Kushuaha et al., 1976) as phosphatidic acid is a common precursor to both classes. However, little is known about the interaction between these two groups during fungal growth.

(ii) Fatty acids

The main unsaturated fatty acids found in A. niger during microcycle conidiation were palmitic acid (C_{16}), stearic acid (C_{18}) and the major saturated acids were oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$). A number of minor components consisting of short carbon chain lengths were also detected in some samples, particularly in conidial extracts. These results are in general agreement with the fatty acid content for A. niger mycelia and conidia previously reported (see Table 4.2). However, as shown in Fig. 4.11 and 4.13, there is a much lower percentage of palmitic acid in the conidia than that previously reported by Gunasekaran et al. (1972c).

The giant cells at 24h, which may be taken as analagous to mycelial growth contain very little detectable linolenic acid whereas Gunasekaran et al. (1972c) reported a content of 15.6% which again is in contrast to the zero level observed by Farag et al. (1981).

There is a more pronounced change in the fatty acids of the total lipid fraction than in the triacylglycerol fraction during the preliminary twelve hour period (0 - 12h). Percentage levels of palmitic acid in the total lipid increase markedly accompanied by a rapid decline in oleic acid levels, and although these changes are echoed in the triacylglycerol fraction, the trends are less dramatic. Thus the degree of saturation remained quite constant over the 0 - 12h period in the triacylglycerol fraction, but increased in the total lipid extracted. This suggests a change in saturation of the other principal components of the total lipid, i.e. the polar fraction, occurring during this period of spore germination and the initial stages of giant cell formation.

During the final twelve hour period of giant cell formation with the temperature increased to 44^{o}C, little change in the degree of saturation of the fatty acids in the total lipid was recorded. However, a marked increase in saturation of the fatty acids of the triacylglycerol fraction was noted over this period. This indicates a dramatic decrease in saturation in the other components of the total lipid fraction e.g. polar lipid, so that the increase in saturation of the triacylglycerol fraction is completely masked.

The lowering of the temperature to 30^{o}C allowing conidiophore production is accompanied by an increase in unsaturation as recorded in both the total lipid and triacylglycerol fractions. The increase is mainly due to linoleic acid.

The percentage of unsaturated fatty acids continued to increase in the triacylglycerol fraction to 48h, but remained constant in the total lipid fraction. This indicates a trend towards saturation in one or more of the other lipid classes e.g. polar lipids, sterol esters.

The fatty acids in the total lipid fraction is the result of hydrolysis of triacylglycerols, sterol esters or polar lipids. A small amount of free fatty acids are also present in the cells but would not be quantified by the method utilised. The changes in percentage levels of the fatty acids in the total lipid fraction would therefore, reflect changes in one or all of these lipid classes. The changes in the relative percentage of fatty acids might represent interconversion of the different fatty acids or it could be a reflection of the rate of de novo synthesis and degradation of the various fatty acid or lipid classes.

The percentage content of stearic, oleic and linoleic acids found in conidia were slightly higher in the present study than previously reported by Gunasekaran et al. (1972c). Linolenic acid was reported at 1.4% in the conidia in the present work but none was recorded by Gunasekaran et al. (1972c). The fatty acid percentage levels found in the mycelia of A. niger have been previously reported by Weete (1980); Farag et al. (1981) and Stretton et al. (1976). The palmitic acid levels vary between 15.8 and 20.5% in these reports which compares to 17.7 to 29.1% over microcycle growth. There is a great variety in the stearic acid levels of A. niger mycelia with values of 0.3, 7.2 and 15.1% recorded compared to values of 4.3 to 5.6% in the microcycle conidiation growth forms.

A similar variation was recorded for linolenic acid with values of 0 to 15.6% previously recorded comparing to 0.2 to 3.0% in the present study. The previously recorded values for oleic and linoleic acids show less variation (see Table 4.2).

It is apparent from these comparisons that factors such as cultural conditions, carbon and nitrogen sources, age of the fungus and fungal strains exert an influence on the fatty acid composition. The percentage levels of each fatty acid varies over the microcycle conidiation period and these variations could be due to the change in temperature, age of culture, nutrient supply or stage of the reproductive process.

During the microcycle, the proportions of unsaturated fatty acids in both the total and triacylglycerol lipid extracts was found to change in a pattern correlated to the changes in incubation temperature. Lower temperatures were accompanied by an increase in fatty acid unsaturation as previously observed for mycelial growth in A. niger (Pearson and Raper, 1927). Previous observations on thermophilic and mesophilic fungi have revealed that thermophiles contain no appreciable amounts of linolenic acid (Mumma et al., 1970). Thus temperature might exert an influence on the presence of linolenic acid in microcycle conidiation. Although linolenic acid was a minor component contributing no more than 3% towards the total fatty acid content, its percentage levels were higher in the conidia and during the conidiophore development and production stages between 24 and 48h.

The fatty acids of thermophilic fungi have been reported to be more saturated than the corresponding mesophilic species - mesophiles were selected for their morphological similarity to the thermophiles e.g. the thermophile Penicillium duponti was compared to the mesophile Penicillium chrysogenum (Mumma et al., 1970). Also the lipids in some Mucorales were significantly less saturated in the thermophiles (Sumner et al., 1969). The high incubation temperatures employed during giant cell formation may thus have a relevance to the degree of unsaturation.

The degree of unsaturation (double bonds/mole) of the fatty acids of A. niger cells undergoing microcycle conidiation are given in Table 4.4. These values were calculated from the data in Figs. 4.11, 4.12, 4.13 and 4.14 according to Weete, 1980, and can be compared to values for the total lipid degree of unsaturation in other fungi previously reported (see Table 4.6). The fungi grown at lower temperatures exhibit a greater degree of lipid unsaturation, a trend which is apparent during the conidiophore development stage of microcycle conidiation (24 - 48h) when the culture is incubated at the lower temperature of 30^{o}C. Conversely, the lowest degree of unsaturation of the fatty acids of the A. niger cells was noted during growth at 44^{o}C, these results being in agreement with the values obtained by previous workers growing fungi at higher temperatures (Table 4.6).

It has also been previously reported that the lipids of mesophilic fungi, grown at their optimum growth temperature, have a high degree of unsaturation (0.96 to 1.60) compared to that of thermophilic fungi (0.65 to 1.01) (Mumma et al., 1970).

The desaturase enzyme is oxygen dependant (Weete, 1980) and it is possible that the temperature effect on unsaturation, although biologically valuable, may be explained by the decreased solubility of oxygen in the media at elevated temperatures.

Fungus	Unsaturation (Δ /mole)	Temperature (^{o}C)	Reference
Candida scottii (AL25)	1.72	10	Kates & Baxter, 1962
Candida utilis NCYC 321 (mesophile)	1.51	15	Brown & Rose, 1969
Sclerotinia sclerotiorum (14 day old)	2.09	5	Sumner & Colotelo, 1970
Rhizopus arrhizus	0.85 1.29	15 30	Gunasekaran et al., 1972a
Penicillium chrysogenum	1.60	25	Mumma et al., 1970
Pythium ultimum	0.82	30	Chang & Matson, 1972
Penicillium duponti (thermophile)	0.86	45	Mumma et al., 1970
Rhizopus sp.	0.94	48	Sumner & Morgan, 1969

Table 4.6 The effect of growth temperature on the degree of unsaturation of fungal lipids.

5. LIPASE AND ESTERASE ACTIVITY DURING MICROCYCLE CONIDIATION IN ASPERGILLUS NIGER

5.1 INTRODUCTION

Lipolytic enzymes are necessary for the biological turnover of lipids and for their re-utilization. They are required as digestive enzymes and control the accumulation of individual products and the utilization of stored reserves. The enzymes are involved in the metabolism of intracellular lipids and hence in the functioning of biological membranes. Other specific actions of various lipolytic enzymes contribute to their importance in the medical, therapeutic and food industries.

Esterases hydrolyse ester bonds in general but lipases form a distinct class which are able to act on insoluble substrates at an interphase between aqueous and lipid phases. Many workers have not differentiated between enzymes which can hydrolyse water soluble esters and true lipases which act at an oil/water interface. The initial stage in hydrolysis is the splitting of fatty acids esterified to the primary hydroxyls of glycerol. The most extensively studied lipase, the animal pancreatic lipase has a high reactivity to ester bonds at the C-1 and C-3 positions of triacylglycerols. The fatty acids in these positions are initially removed at the same rates, but the hydrolysis of subsequently produced diacylglycerols and monoacylglycerols proceeds more slowly. The preferential removal of the C-1 and C-3 fatty acids, together with the slowing down of the reaction at the final stages leads to the accumulation of the monoacylglycerol as the primary product. This is especially the case *in vivo* where the re-synthesis of di- and tri-acylglycerols via the monoglyceride pathway may regulate the extent of hydrolysis.

Lipases, in addition to their occurrence in the digestive system of animals, are widely distributed in all other organisms. They are predominant where there is an obvious need for such enzymes e.g. in oil-rich seeds or in micro-organisms growing on lipid based media. In plants lipases have been widely reported in seed tissues and research has centred around the castor bean lipase. Seeds are usually rich in triacylglycerols which serve as a compact source of energy for germination. The crushing or storage of seeds generally activates dormant lipases and problems can arise in the commercial use of such seeds. For example, the accumulation of free fatty acids in vegetable oils extracted from seeds can cause the product to become unacceptable and further processing would be required to remove the "contaminants".

Historically, interest in the lipolytic enzymes of micro-organisms centred on their food spoilage effects, in particular on dairy products. The glycerols of the higher fatty acids are the major constituents of most natural fats. Lipases release these fatty acids which are then more readily broken down to carbonyls or other volatile compounds responsible for rancidity produced during spoilage. However, in some cases, in particular during cheese production, the free fatty acids contribute to a desirable flavour. For example, Penicillium roqueforti spores are deliberately added in the preparation of Roquefort cheese so that the action of the lipase produced by the fungus can contribute to the distinctive flavour.

Lipases of microbial or fungal origin, but with similar properties to those of the enzymes required in lipid breakdown may, in future, be used to control malfunctions of fat metabolism. Such advances might help curb the increase in cardio-vascular diseases in humans.

Bacteria do not usually store triacylglycerol, and therefore, in contrast to plants, their lipases function in the breakdown of exogenous triacylglycerols. Thus mircobial lipases are not membrane bound but are soluble enzymes of a relatively low molecular weight. Some microbes produce lipases similar to the plant and animal enzymes which tend to accumulate monoacylglycerols. However, rumen microbes convert triacylglycerols rapidly to fatty acids without accumulation of intermediary products.

Yeast and fungi contain more triacylglycerols than bacteria, but in many cases the contents are minor in comparison to phospholipids. These organisms contain both intracellular lipases which may function in spore germination and extracellular lipases important in such roles as the degradation of soil triacylglycerols of plant and animal origin. Most fungal lipases are relatively stable as compared with their animal and plant counterparts and some are inducible.

Numerous fungal species have been tested for lipase activity by Alford et al. (1964) and nine of these including Aspergillus flavus were considered lipase producers. Other fungi in which lipase activity has been reported include Mucor pusillus (Somkuti & Babel, 1968; Somkuti et al., 1969), Candida cylindricaceae (Yamada & Machida, 1962), Torulopsis ernobii (Yoshida et al., 1968), and Puccinia graminis tritici (Knoche & Horner, 1970). The most extensively studied fungal lipase is that of Rhizopus arrhizus (Labourer and Labrousse, 1964; 1966; 1968) which is used as an industrial source of the enzyme.

Most fungal lipases preferentially cleave fatty acids from the C-1 position of triacylglycerols, although a lipase from Aspergillus flavus shows no positional specificity (Weete, 1980). Fukumoto et al. (1963) purified a lipase from A. niger which also appeared to hydrolyse triacylglycerol without positional specificity. In contrast, a lipase from Geotrichum candidum shows the greatest degree of specificity of all studied lipases in that it cleaves at the Δ^9- unsaturation carbon regardless of its position on the triacylglycerol molecule.

The fluctuations in triacylglycerol levels over the 48h period of microcycle conidiation in A. niger indicate the presence of a hydrolysing enzyme such as a lipase. Esterase activity has been previously reported in vegetative mycelial growth of A. niger and was assumed to indicate lipase activity (Lloyd et al., 1971;1972). Furthermore, lipase activity has been reported for various strains of A. niger, e.g. A. niger ICA-34 grown on food industry wastes (Birnescu & Vasu, 1982); fat hydrolysis was noted when a lipase preparation from A. niger was added to tallow, coconut oil and olive oil (Linfield et al., 1984), and a lipase preparation from A. niger catalyzed the synthesis of glycerides from glycerol and fatty acids in addition to their hydrolysis (Okumara, 1982). Fukumoto et al., 1963 found that a strain of A. niger selected by extensive screening secreted a large amount of lipase when grown on a bran-koji medium.

Volkova and Lebedeva (1979) screened 35 strains of the genus Aspergillus belonging to 35 species and found only five, including A. niger exhibiting lipolytic activity which was unstable and disappeared on the second to third day of growth. A further 10

strains showed a weak lipolytic activity, but detectable lipase was recorded for the remaining 20 strains. Esterase activity has been previously reported for A. niger (Lloyd et al., 1971;1972). The esterase was detected at a low basal level in axenic cultures but greater activity was induced in conidiating samples. A very rapid increase in esterase activity was reported during differentiation of conidiophore tips, and vesicle, phialide and conidia formation. Also, the periods of highest and lowest cell lipid content during vegetative growth and conidiation corresponded to the periods of lowest and highest esterase activity respectively. This suggests a role for the esterases in the breakdown of lipid reserves (Lloyd et al., 1971) and they may also be synthesized in the phialides during conidia formation and serve in the mobilization of lipid reserves during the subsequent germination of the conidia (Lloyd et al., 1972).

The present work measured triacylglycerol lipase and water-soluble esterase activity in order to investigate their relationship to the observed changes in lipid levels which occur during microcycle conidiation.

5.2 RESULTS

5.2.1 Lipase Activity

Samples of intact and homogenised A. niger cells were tested for lipase (E.C.3.1.1.3) activity at various stages throughout microcycle conidiation as detailed in Chapter 2.16.

The enzyme activity was estimated using a range of buffer solutions in an attempt to determine the pH optimum of any A. niger microcycle lipases detected. The analysis depended on the release, by lipolytic activity, of radiolabelled free fatty acids from a ^{14}C-labelled triolein substrate. A commercially available fungal lipase from Candida cylindracea was used to prepare a calibration curve and to test the assay methods. The lipase activity was tested using concentrated A. niger cells as the enzyme source at various stages of microcycle conidiation. The results obtained showed much variation and were at the lower limits detectable by the assay method. However, the effectiveness of the assay method is apparent from the results obtained using the Candida lipase (Fig 2.4).

The results obtained are illustrated in Figs 5.1 and 5.2. The graphs show peaks of lipase activity at pH 8.0 for A. niger cells at the 12h stage in the microcycle and at pH 7.0 for cells at 36h. A smaller peak in lipase activity was also evident at pH 7.0 for the 12h cell samples. Some lipase activity occurred at pH 7.0 in the 24h cell sample, but the nmoles of fatty acid released was lower than at 12h and 36h. High lipase activity was apparent at 12 and 36h at pH 7.5, but much lower lipolytic activity occurred at pH 5.0, 6.0 and 9.0.

Although lipase activity in the 36h cell sample reached a peak of 1.66 nmoles fatty acid released per 1×10^6 cells, the activity dropped to 0.88 nmoles at pH 7.5 to 0.14 nmoles at pH 8.0. However at 42h there was an increase in lipase activity at pH 7.5 to 8.0 as compared to that obtained at pH 7.0.

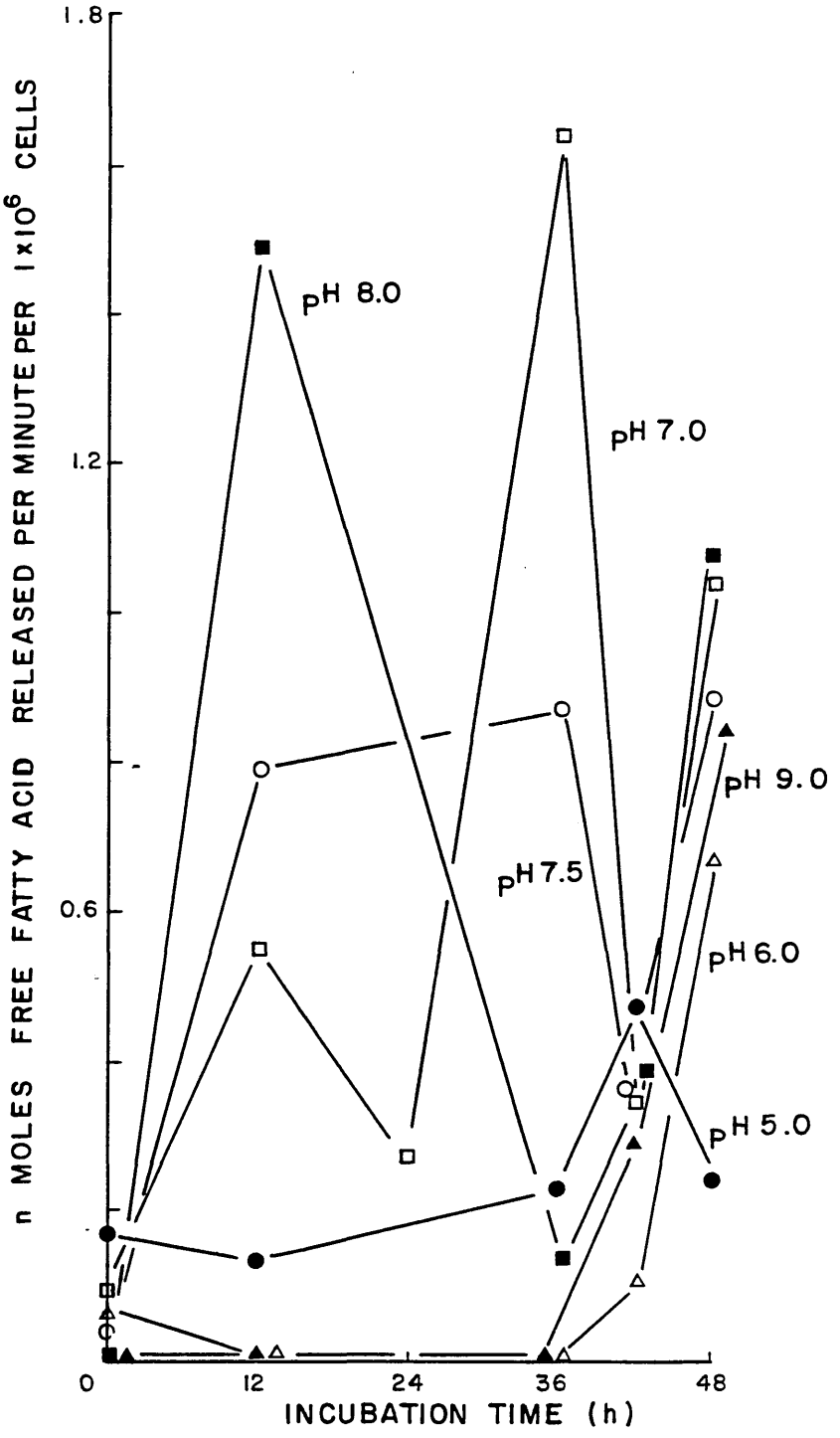


FIG.5.1 LIPASE ACTIVITY DURING MICRO-CYCLE CONIDIATION IN A. NIGER OVER A $_{p}$H RANGE 5.0 - 9.0.

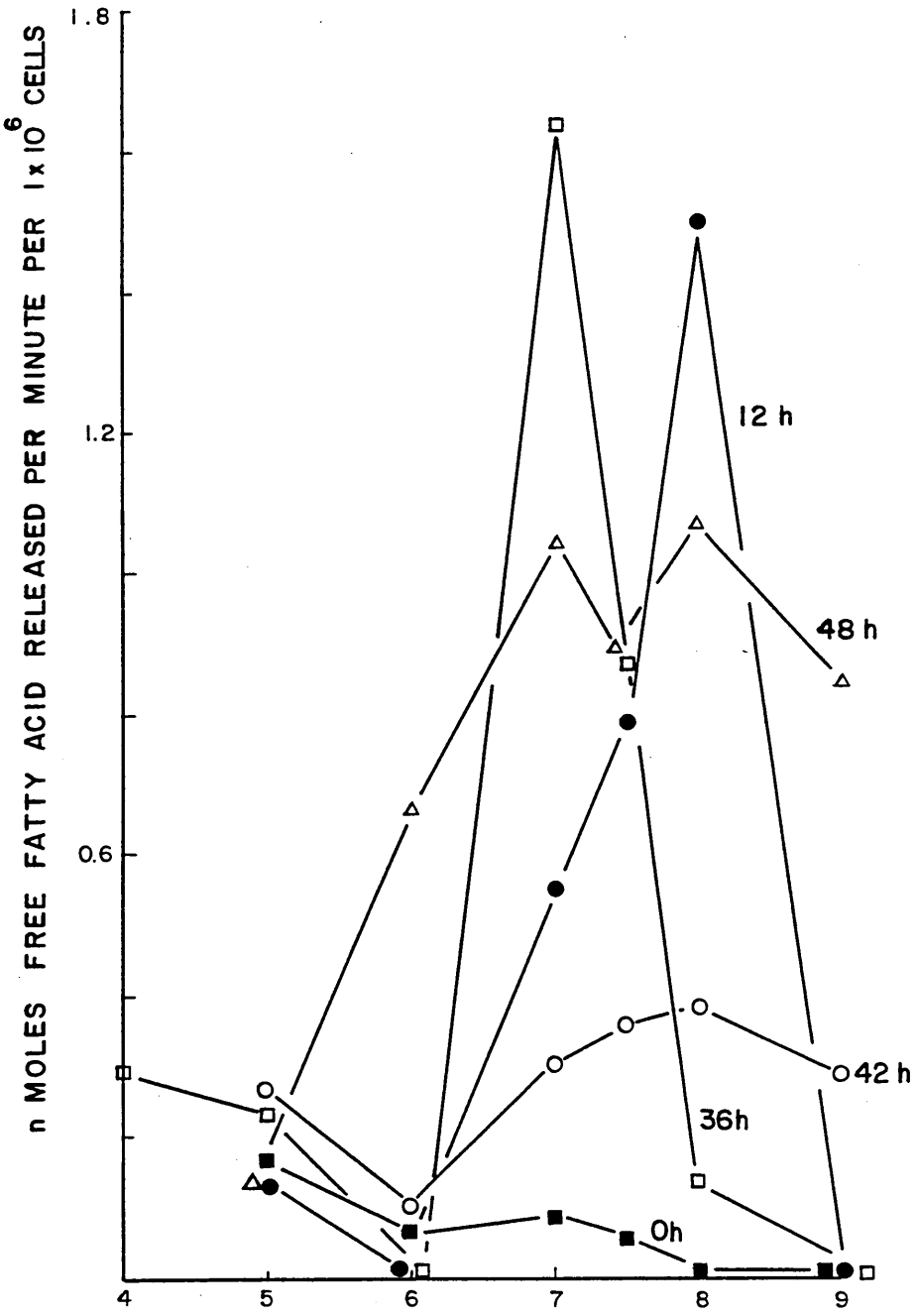


FIG.5.2 LIPASE ACTIVITY THROUGHOUT MICROCYCLE CONIDIATION OF A. NIGER OVER A pH RANGE 5.0 - 9.0

At 48h, lipases were active in a range of pH values from 6.0 to 9.0. The 48h cells were unique in the microcycle in having a high lipase activity at pH 6.0, although some lipolytic activity was evident in all cell samples tested at pH 5.0.

The A. niger growth medium was also tested for lipase activity over the 48h microcycle. In all cases, with the exception of the values recorded at 36h for pH 4.0, 7.0 and 9.0, the results obtained, were equal to or less than the blank values. For the 36h medium sample tested at pH 4.0, lipase activity resulted in 0.13 nmoles of free fatty acid being released per min per ml of medium. At pH 7.0, 0.14 nmoles of free fatty acid were released decreasing to 0.08 nmoles at pH 9.0.

5.2.2 Esterase Activity

The esterase (E.C.3.1.1.1) activity of A. niger cells undergoing microcycle conidiation was assayed as reported in Chapter 2.16.2. Both homogenised and intact cells were tested for enzyme activity which was expressed as nmoles 1-naphthol released per 1 x 10^6 cells, as illustrated in Fig 5.3.

The esterase activity detected in the A. niger spores was relatively low, but increased steadily throughout microcycle conidiation. However, the amount of esterase activity from 0 - 36h was in the range 0.68 to 4.03 nmoles 1-naphthol produced per 1 x 10^6 cells, but a dramatic increase in activity to 18.94 nmoles occurred from 36 - 48h.

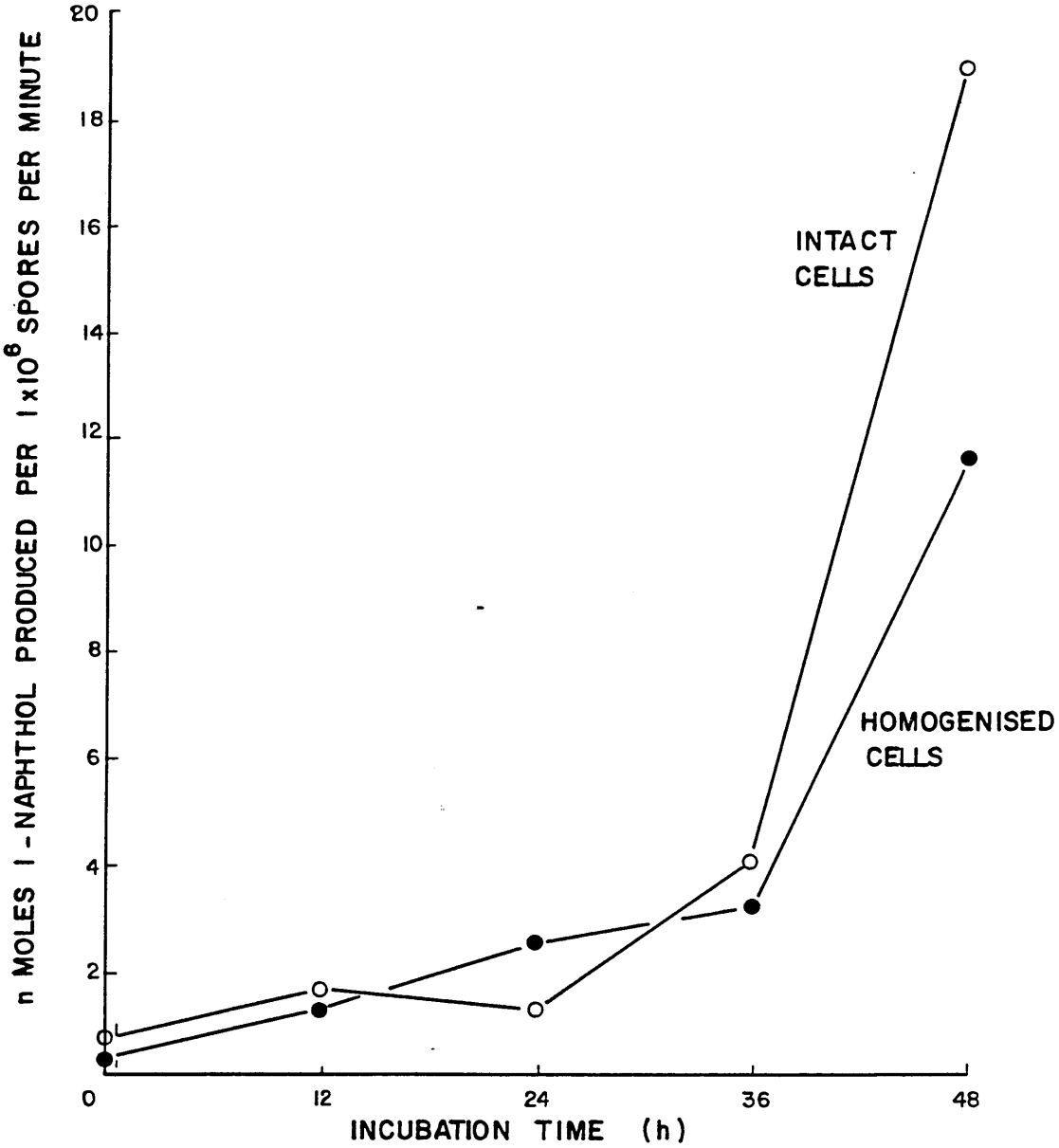


FIG.5.3 ESTERASE ACTIVITY DURING MICROCYCLE IN A.NIGER IN INTACT CELLS & HOMOGENISED CELLS.

The esterase activity recorded in the medium was 3.33 nmoles 1-naphthol produced per 1ml of medium at 12h rising to 3.47 nmoles from 24 - 48h. In general there was more esterase activity detected in entire A. niger cells as compared to homogenised samples. This, however, was not the case at 24h where there was greater activity in the homogenised cell sample as opposed to the intact cells.

5.3 DISCUSSION

The lipase assay was conducted over a range of pH values as the optimal pH for A. niger lipase was unknown. In general, microbial lipases are active over a wide pH range but in plant seed tissues two distinct types of lipases have been reported, one having an acid pH optimum, the other an alkali (Harwood & Russell, 1984). Fungal lipases usually have a pH optimum of 8.0 (Weete, 1980) but much variation has been reported e.g. Mucor pusillus (Somkuti & Babel, 1968; Somkuti et al., 1969) has an optimum of pH 5.5 and Puccinia graminis tritci an optimum of pH 6.7 (Knoche & Horner, 1970).

A low level of lipase activity was recorded in the ungerminated spores of A. niger. However a marked increase in the lipase present occurred during the first 12h of spore germination and giant cell formation suggesting the utilization of triacylglycerols at these stages releasing free fatty acids for oxidation. There are varying reports as to the utilization of lipids during spore germination in fungi (See Chapter 3.1). Lipase has been isolated from germinating uredeospores of Uromyces phaseoli (Knoche & Horner, 1970).

The rapid decrease in phospholipid content during the very early stage of germination in A. niger conidia has been reported (Nishi, 1961) and the activity of phospholipase may be responsible. The phospholipid catabolism in A. niger was also accompanied by an increase in sugar phosphates and nucleotides with the incorporation of phosphorous into RNA. In the present study a low phospholipid level (reported as polar lipid in Fig. 4.7) was detected in ungerminated spores. However, the phospholipid content increased during giant cell growth reaching 9.84 µg per 1 x 10^6 cells at 24h. During the subsequent eight hours, through the initial stages of conidiophore emergence and development, the phospholipid level decreased to 4.85 µg per 1 x 10^6 cells. The presence of a greater phospholipase activity may therefore be expected from 24 - 32h in the microcycle and this may in part explain the increase in esterase activity recorded from 24 - 36h, if such a phospholipase is able to utilize water soluble esters as an alternative artificial substrate.

The relatively low level of lipase activity recorded at 24 and 42h in the microcycle probably corresponds to periods of lipid accumulation (See Fig. 4.5). The peak of lipase activity at 36h for medium lipases may be explained by the catabolism of triacylglycerols for the energy demanding processes involved in conidia production. It is interesting to note that the only significant presence of lipase in the culture medium throughout the microcycle occurred at 36h. However, although lipase was detected at pH 7.0 in the medium which corresponds to a peak in cellular lipase, the enzyme was also present at pH 9.0 in the medium but none was detected in the cells at this pH. The increase in cellular lipase at 48h may be due to the utilization of internal lipid reserves in the cell due to an exhaustion of exogenous substrates.

It has been reported that the Mucor javanicus lipase is influenced by the degree of unsaturation in the substrate (Ogiso & Sugiura, 1971). The hydrolyses of triacylglycerols containing oleic and linoleic acids are more readily catalyzed than those with higher levels of linolenic acid (Weete, 1980). However, during the present study very low levels of linolenic acid (0.05 - 0.54% of total fatty acid content) were present in the triacylglycerols throughout microcycle conidiation. Thus the interference due to the increased number of double bonds causing the chain to bend making the development of the enzyme/substrate complex difficult would not be expected to be a major factor influencing lipase activity in the microcycle.

Esterases have been previously reported in A. niger van Tiegham, but only at the late stages on conidiation, in normal asexual reproduction (Fig 3.1) when intracellular lipid decreased (Lloyd et al., 1971). However, in the present study, esterase was detected in the cells throughout microcycle conidiation but with a very marked increase during the period of conidiophore maturation and conidial production. Lloyd et al. (1971), detected no esterase in the cell-free growth medium of A. niger undergoing normal asexual reproduction in contrast to the relatively high esterase activity in the present study on microcycle conidiation. The assay employed would detect any enzyme hydrolysing ester bonds and could include true lipases which normally work at the lipid/water if they can accept an artificial water-soluble substrate. It is apparent from the results in Section 5.2.1 that the esterase detected in the culture medium during microcycle conidiation is not a true lipase, and the intracellular esterase may also be distinct from true lipase activity.

The intact giant cells generally showed greater esterase activity when compared to their homogenised equivalents. The indication is that the homogenisation treatment inactivates some of the esterases. However, at 24h when the giant cells have achieved maximum spherical growth a greater esterase presence was noted in the homogenised cells. At this stage the cell wall is extremely thick measuring up to 2 µm wide (Smith et al., 1981a) and some cell wall breakdown may be essential for the detection of intracellular esterases. An increase in esterase activity was noted during the first 12h of spherical giant cell growth. The enzymatic activity at this stage could be due to the utilization of stored cellular materials during spore germination and giant cell development.

There was an overall increase in esterase activity throughout the rest of the microcycle with the exception of the apparent decrease in the esterase present in the intact cells at 24h. If this is assumed to be a masking effect due to the extremely thick cell wall present at this stage, then a gradual increase in esterase activity was evident during the final stages of spherical giant cell growth and through the early stages of conidiophore production. A very pronounced increase in the esterase present occurred during the final stages of conidiophore development and conidia production. At this stage, there was a decrease in the cell lipid content corresponding to the period of greater energy demands for conidiation. It also coincides with the period of rapid decline in the glucose substrate in the growth medium, so increasing the demand for an intracellular energy source. The esterase activity in the growth medium was found to remain relatively constant throughout the microcycle.

The esterase activity found in the present study on A. niger can be compared to those previously recorded by Lloyd et al., 1972. At the stage of conidiophore initiation and growth (0-32h) during the normal asexual reproduction cycle studied by Lloyd et al., 1972, values of 4×10^3 to 8×10^3 mg 1-naphthol released per mg protein were obtained for esterase activity. These are much higher than the values of 0.09×10^3 to 0.19×10^3 mg 1-naphthol released per mg protein recorded for the comparable period (24-36h) during microcycle conidiation in the present study.

6. INVESTIGATION INTO THE FATE OF RADIOLABELLED CARBON SUBSTRATES SUPPLIED IN THE GROWTH MEDIUM DURING MICROCYCLE CONIDIATION IN A. NIGER

6.1 INTRODUCTION

A number of other workers have previously undertaken studies on various biochemical aspects of microcycle conidiation. These have included the changes in cell wall components throughout the microcycle (Deans, 1978), various cellular analyses (Yahya, 1979; Duncan *et al.*, 1978), the effect of known phosphodiesterase inhibitors (Yahya, 1979) and the formation of viable protoplast from giant cells (Davis *et al.*, 1977; Yahya, 1979; Smith *et al.*, 1981a). Although the total amount of lipid present in the cell wall has been previously investigated throughout microcycle conidiation (Deans, 1978), little work has been undertaken on any other aspect of lipid biochemistry in the microcycle.

The results in Chapter 4 indicate that lipids accumulate in the giant cells during spherical growth and that lipid utilisation followed by synthesis occurs during conidiophore production. The fate of radiolabelled precursors added to the growth medium was traced in the present study and the incorporation into the various lipid classes investigated.

The biochemical pathways likely to be operative in this study and which lead to the biosynthesis of the various lipid classes are illustrated in Fig. 6.1. Fatty acids are a major lipid class found in fungi although they tend not to occur in large quantities in the free form. They have a marked affinity for many proteins, and are usually found as esters. The fatty acids are mainly incorporated into the structure of triacylglycerols, phospholipids and sterol esters, as was discussed in Chapter 4.

Acetyl-CoA is the building block for de novo fatty acid synthesis, and the various metabolic pathways leading to its formation are illustrated in Fig. 6.1. The subsequent events leading to fatty acid biosynthesis in fungi and other organisms are illustrated in Fig. 6.2. The enzymes which catalyze the synthesis of fatty acids are collectively known as fatty acid synthetases and in plants and bacteria these occur as seven individual enzymes occurring as a functional aggregate. However, in fungi and animals the fatty acid synthetases occur as multienzyme complexes, with multiple active sites on a single protein chain. The fungal fatty acid synthetases are considerably larger molecules than those of animal origin. The yeast fatty acid synthetase has been extensively studied and the subject is reviewed in Weete (1980). Acetyl CoA carboxylase is considered important in the regulation of fatty acid synthesis and it has been extensively studied in various yeasts (Weete, 1980). Fatty acid biosynthesis results in the formation of fatty acids with chain lengths of up to sixteen carbon atoms, and elongation beyond this is achieved either by the condensation of acetyl-CoA with a pre-formed acyl-CoA or by using malonyl-CoA.

Fig. 6.1 illustrates that all three carbon substrates used in the growth medium for microcycle conidiation viz: glucose, alanine and glutamic acid can be utilised as precursors of acetyl CoA and hence fatty acids.

The metabolism of sugar or sugar derivatives may occur by three pathways. The Embden-Meyerhof-Parnas (E.M.P.) pathway is the most common means of energy production in fungi.

The pentose-phosphate (P.P.) sequence, one of the hexose monophosphate (H.M.P.) pathways is also utilised and is important in supplying $NADPH_2$ instead of $NADH_2$ for a range of biosynthetic reactions occurring during differentiation, and also for producing ribose as a precursor of nucleic acids. The third pathway, namely the Enter-Doudoroff (E.D.), has only a restricted occurrence in fungi. The activities of two of the enzymes of the E.D. pathway, 6-phosphogluconate dehydrase and 2 keto-3-deoxy-6 phosphogluconate dehydrase, have been assayed in both sporulating and non-sporulating Aspergillus niger mycelia, but have not been observed in either (Smith, Valenzuela-Perez and Ng, 1971).

Radioisotopes have been used in the investigation of glycolytical pathways and to decide on the mode of carbohydrate metabolism being used by a particular fungus at various stages in its life cycle. Much of the work on glycolysis in fungi has been done by measuring the outputs of radiolabelled carbon dioxide from glucose-1-^{14}C and glucose-6-^{14}C (Cochrane, 1976). In the E.M.P. pathway, carbons 1 and 6 of glucose end up as the methyl group of pyruvate (see Fig. 6.15) and a C1/C6 ratio of unity can be expected. Conversely, in the pentose phosphate pathway, the carbon-1 of glucose is split off in the oxidative decarboxylation of 6-phosphogluconate, and the ratio of C1/C6 is well above unity until the point of complete oxidation of glucose. However, pentose phosphate formed in the PP pathway is converted to fructose-6-phosphate (F-6-P) and glucose-3-phosphate (G-3-P). The F-6-P formed is thought to be in equilibrium with G-6-P, but if recycling of pentoses to G-6-P occurs, there will be a dilution of the G-6-P content by unlabelled compound arising from the PP pathway.

Thus there is a need to supplement the use of radiotracers with other kinds of analyses. In the present study uniformly radiolabelled carbon substrates were introduced into the microcycle growth medium along with the spore inoculum. The fate of these substrates was traced throughout the various stages of A. niger microcycle conidiation with particular emphasis on the incorporation of label into the cell lipid.

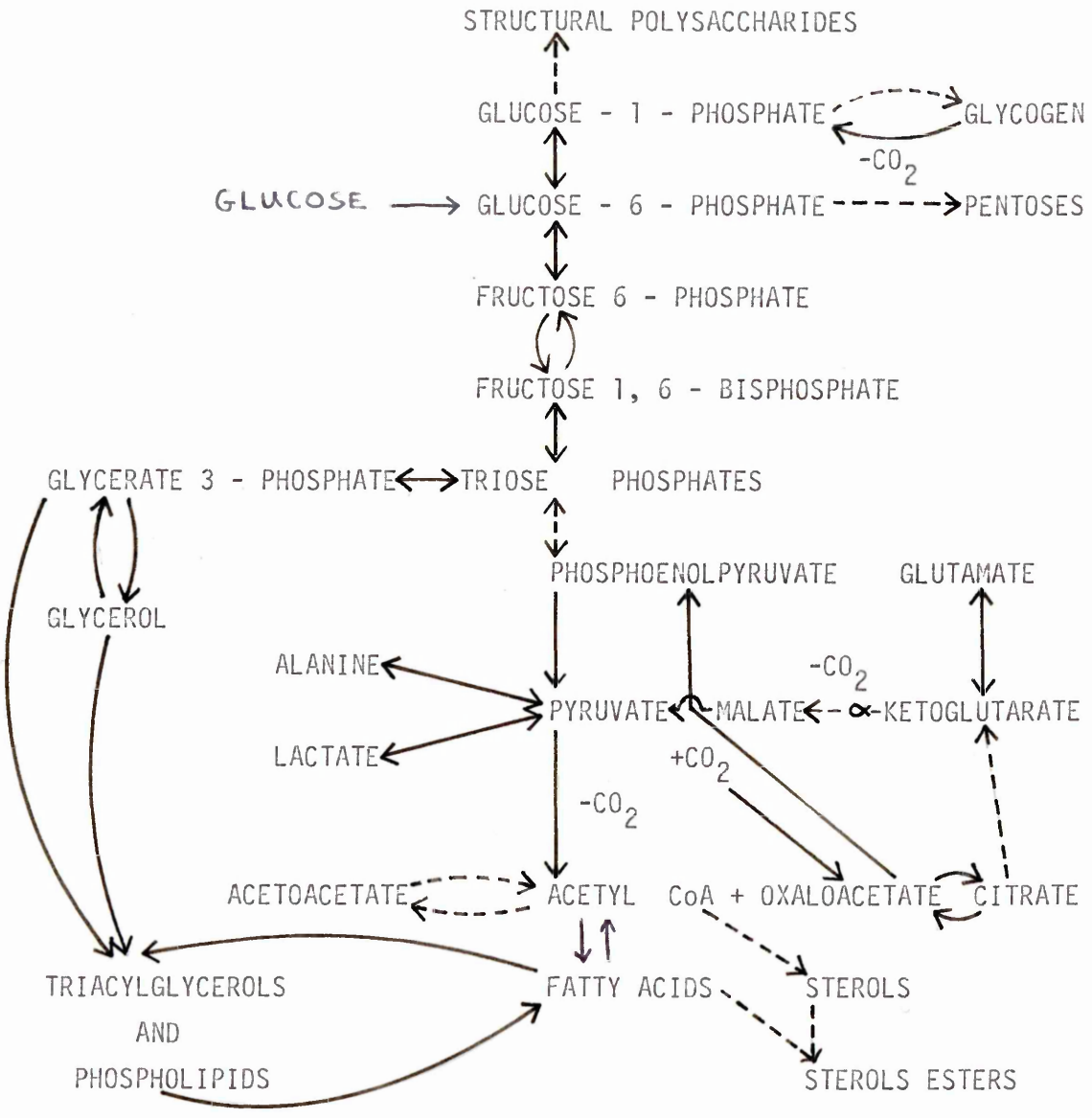


Fig. 6.1 Schematic representations of some of the major transformations occurring in the fungal cell. (White et al., 1978, with modification).

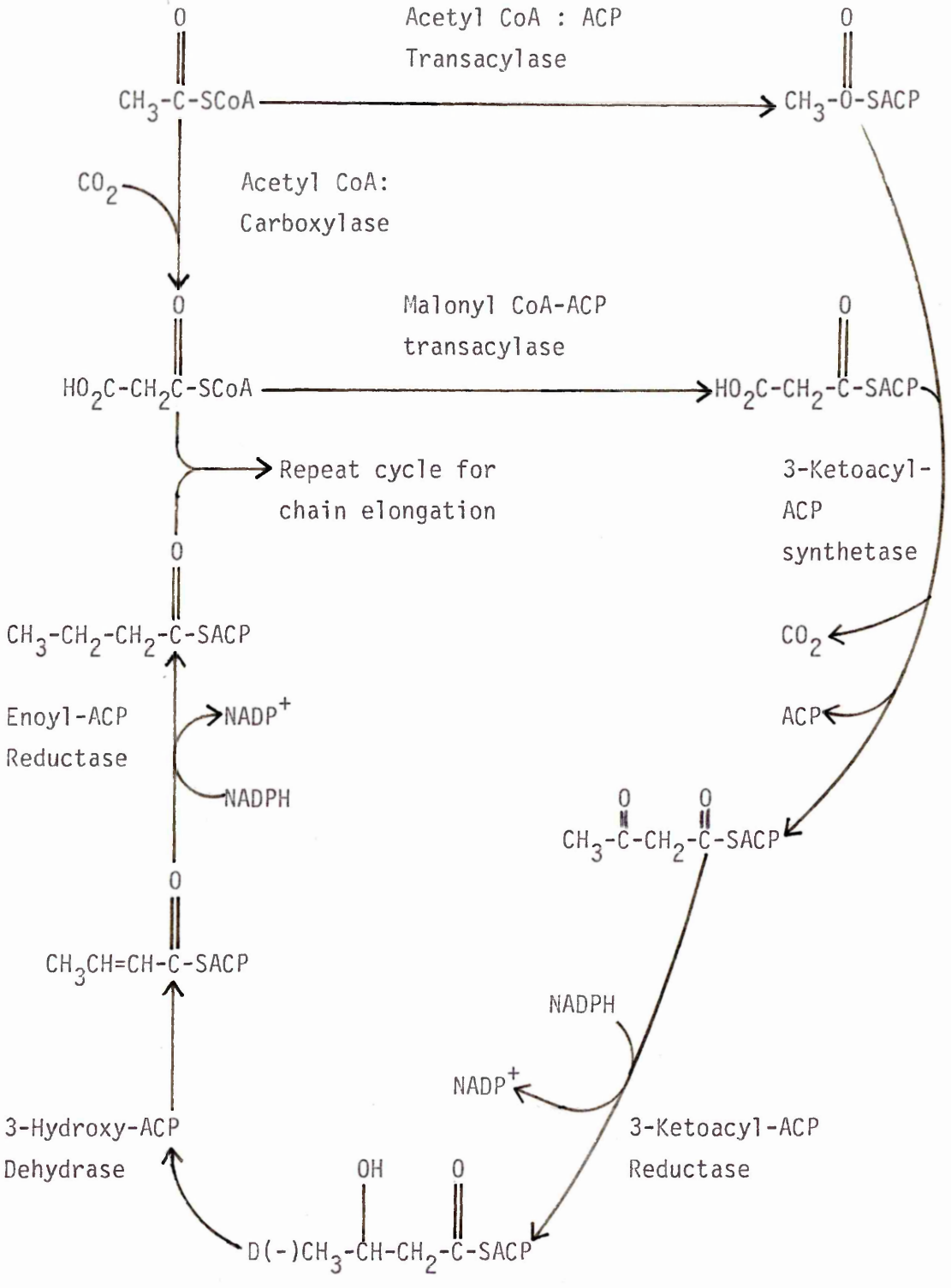


Fig. 6.2 Pathway of de novo fatty acid biosynthesis.
(Weete, 1980).

6.2 RESULTS

Radiolabelled carbon substrates were introduced into the fermenter culture prepared for A. niger microcycle conidiation, as described in Chapter 2.15. The decrease in radiolabelled substrates present in the medium was recorded over the 48h microcycle and the incorporation of the radioisotope were expressed as a percentage of the former in the initial inoculum (Fig. 6.3). Chemical determination of the glucose and total amino content of the incubation medium was also carried out as described in Chapter 2.12 and 13 (Figs. 6.4 and 6.5).

6.2.1 Chemical determination of medium glucose content

The medium glucose content decreased slightly over the first twelve hour period of the microcycle with levels declining from 56.0 to 53.2 µmole glucose per ml of incubation medium. The decline in medium glucose continued over the next twelve hour period decreasing to 34.1 µ moles glucose per ml incubation medium at 24h. Glucose concentration remained at 34.1 µmoles per ml incubation medium during the first four hour period at 30^{o}C but decreased to 28.9 µmoles per ml by 36h. A very marked reduction in glucose medium levels occurred between 36 and 40h with the glucose concentration decreasing to 2.4 µmoles per ml. At 48h, the end of the microcycle, there was no detectable glucose present in the incubation medium.

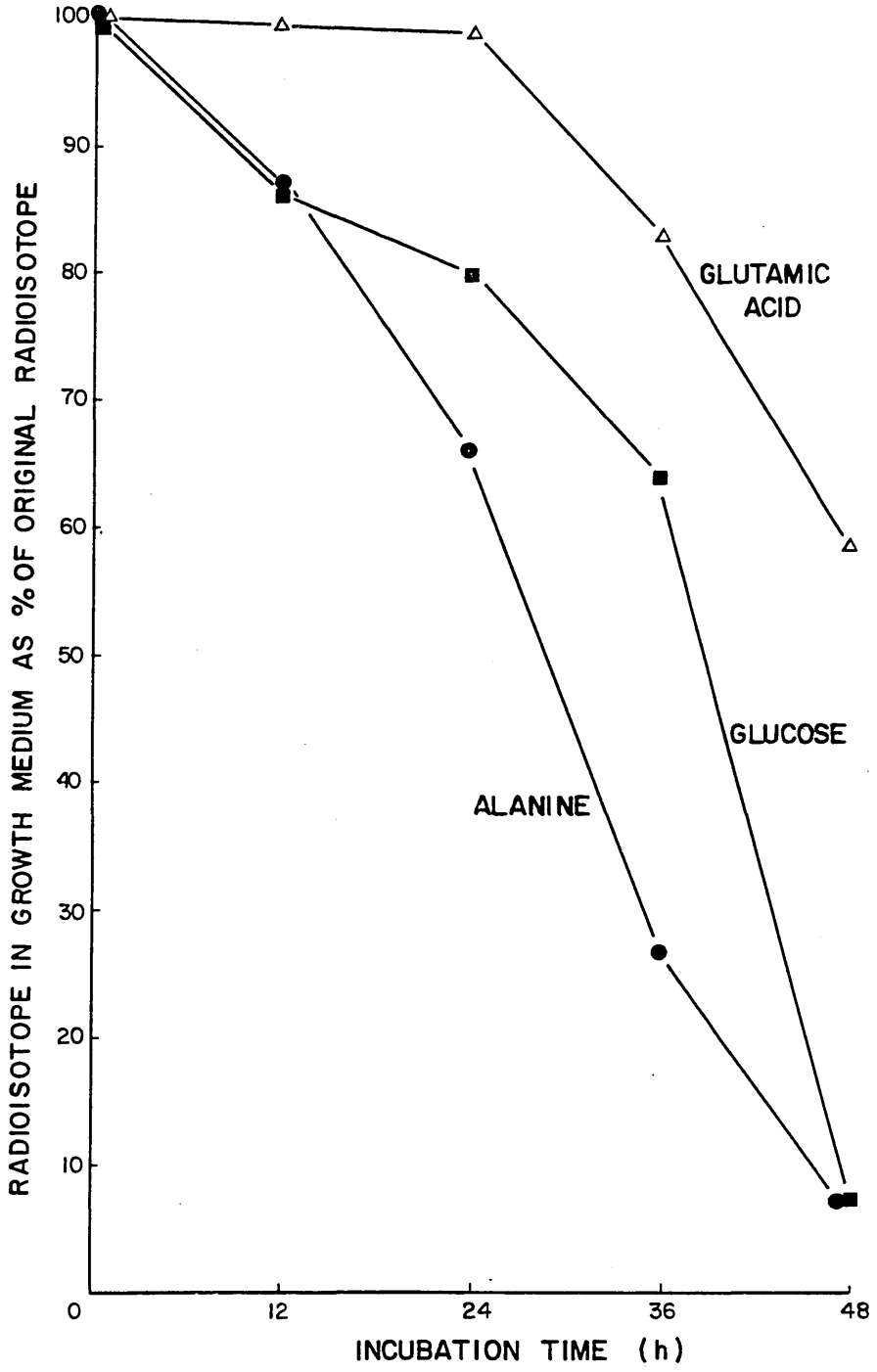


FIG.6.3 RADIOISOTOPE(GLUCOSE, GLUTAMIC ACID & ALANINE) CONTENT OF GROWTH MEDIUM SUPPLIED FOR MICROCYCLE CONIDIATION IN A. NIGER.

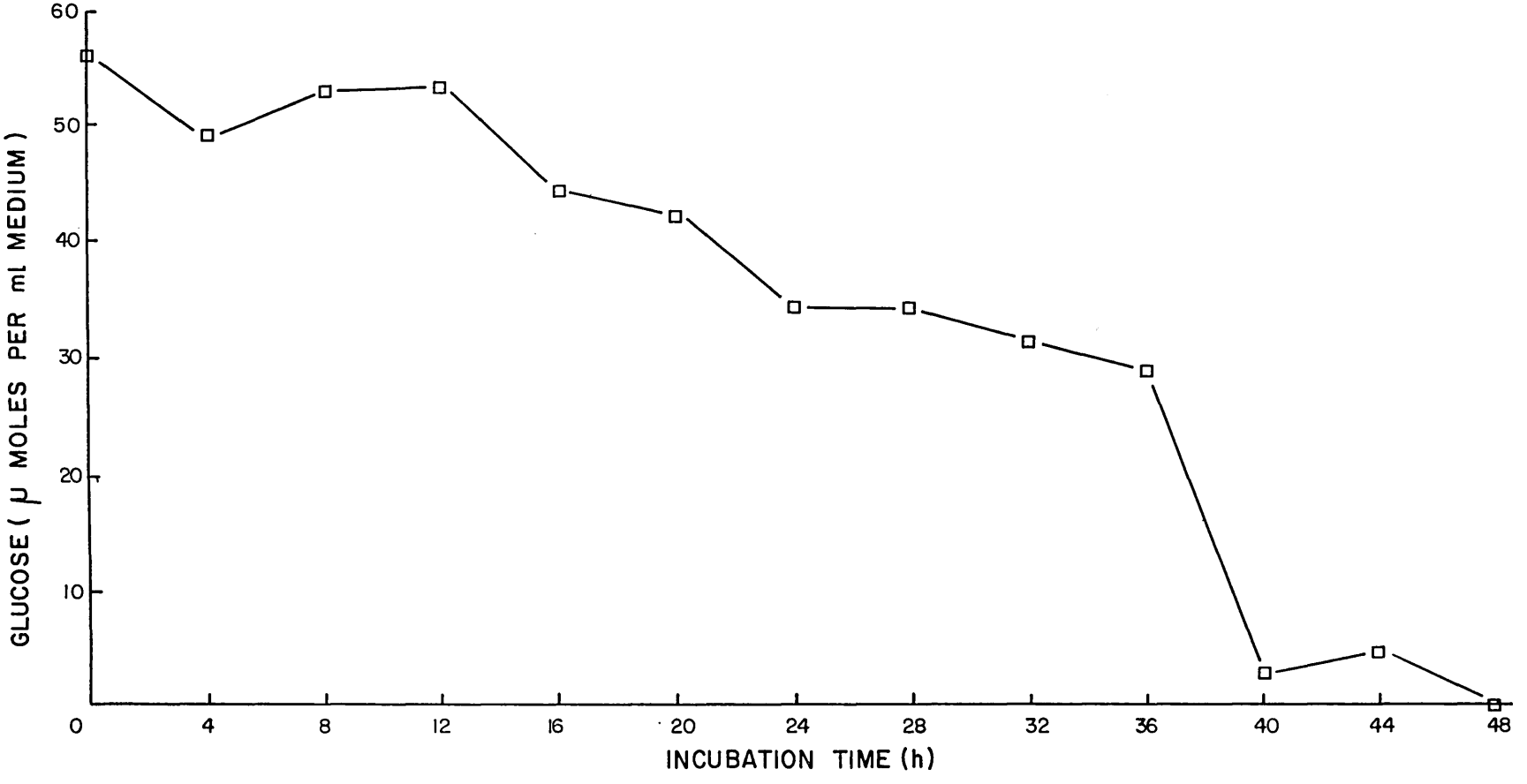


FIG. 6.4 GLUCOSE CONTENT OF GROWTH MEDIUM DURING MICROCYCLE CONIDIATION IN A.NIGER.

6.2.2 Chemical determination of ninhydrin reactive material

There was no detectable change in the amount of ninhydrin reactive material present in the incubation medium throughout the initial 20h of the microcycle. However a decrease from 61 to 54 µmoles of ninhydrin reactive material per ml of incubation medium was noted from 20 to 24h. Following the decrease in temperature to 30°C at 24h, the concentration of ninhydrin reactive material in the medium rose to 77 µmoles per ml at 28h declining to 52 µmoles per ml by 32h. The levels of minhydrin reactive materials remained constant to 36h but then declined steadily reaching 17 µmoles per ml at 48h, the end of the microcycle.

6.2.3 Radiolabelled glucose content

There was a depletion in the glucose content of the medium throughout microcycle conidiation as determined using radioisotope tracers. There was a steady decline in the medium glucose level during the initial 12h stage of giant cell formation where glucose uptake by the cells amounted to 13.0% of the original supplied. During the second stage of spherical growth to 24h, less glucose was taken up by the cells, only amounting to 6.7% of the original supplied at the inoculation stage. The rate of medium glucose depletion increased in the later stages of the microcycle during the periods of conidiophore production and development. During the 24-36h period, 15.4% of the glucose available in the initial incubation medium was taken up by the cells (Fig. 6.3).

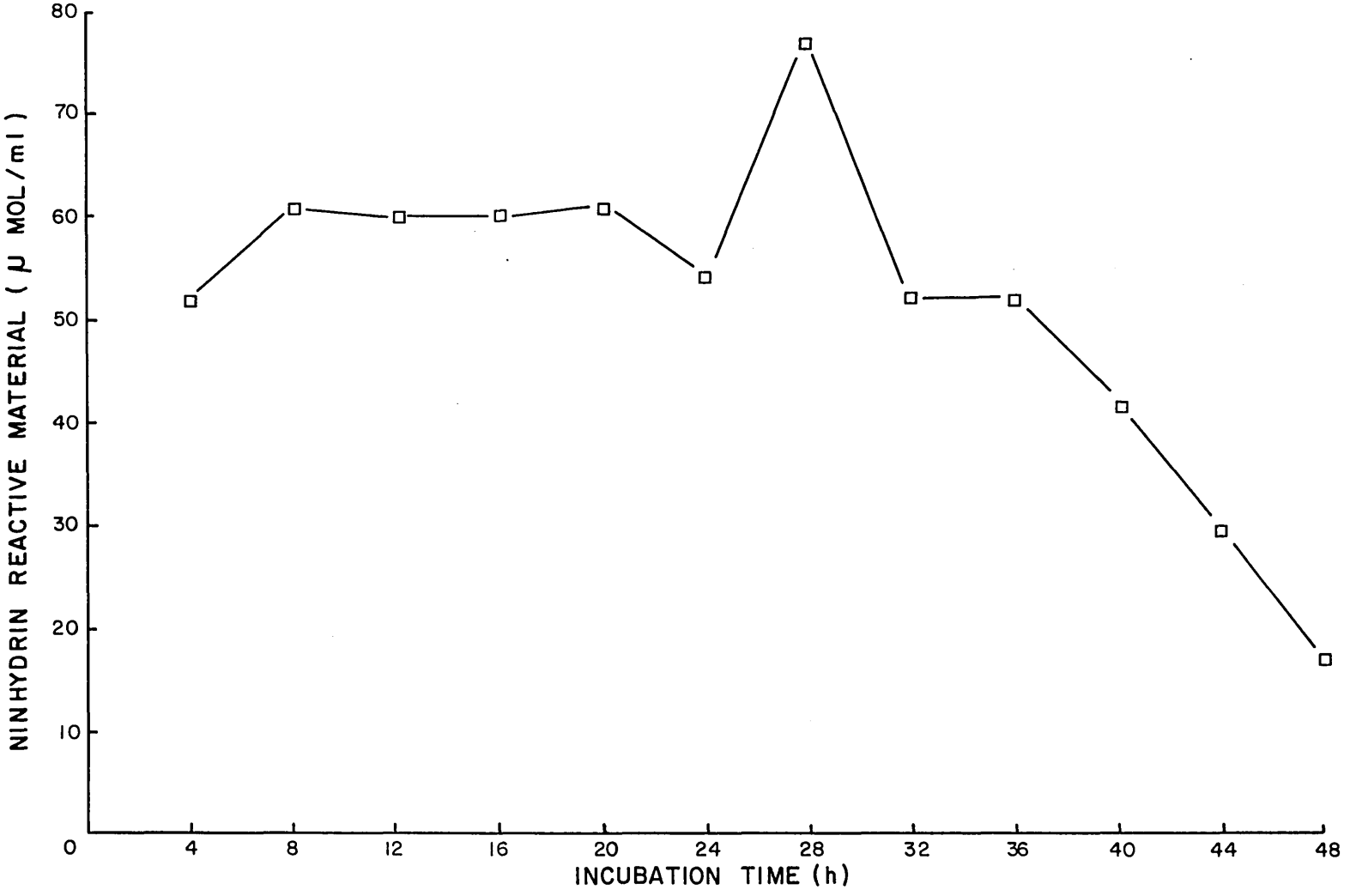


FIG. 6.5 NINHYDRIN REACTIVE MATERIAL PRESENT IN THE GROWTH MEDIUM OF A.NIGER CELLS UNDERGOING MICROCYCLE CONIDIATION

A very marked decline in the glucose present in the medium occurred during the final stages of conidiophore production and conidial development with 56.0% of the original glucose being taken up during this period. Finally, at 48h, only 8% of the original glucose available in the medium remained.

6.2.4 Radiolabelled alanine content

Alanine present in the growth medium decreased rapidly throughout the entire microcycle with only 7% remaining at 48h. During the initial stage of giant cell formation in the 0-12h period, 13.1% of the available alanine was taken up by the cells with an increased uptake of 20.9% during the 12-24h stage of spherical growth. The most marked decline in alanine levels in the growth medium occurred during conidiophore production when an uptake of 39.4% of the original alanine was noted. Over the final twelve hour period of the microcycle, 19.3% of the alanine originally supplied was taken up by the cells leaving 7.3% in the growth medium on completion of the cycle (Fig. 6.3).

6.2.5 Radiolabelled glutamic acid content

Only 1.2% of the supplied glutamic acid is utilised in the 0-24h period of giant cell formation, in contrast to the high alanine uptake during this stage. However, a marked depletion in the glutamic acid content of the medium was noted following the lowering of the temperature to $30^{o}C$ at 24h.

Depletion in medium glutamic acid occurred throughout the process of conidiophore production and conidiogenesis with 15.9% of the radioisotope supplied taken up during the 24-36h stage and 24.2% in the final twelve hour period. A relatively high amount of glutamic acid, 58.5% of the original supplied, was found in the growth medium on completion of the forty-eight hour microcycle (Fig. 6.3).

6.2.6 Utilization of radiolabelled substrates

The radiolabelled substrates taken up from the growth medium into the A. niger cells may be utilised by the cell either in the synthesis of new cellular material or they may be completely metabolized to release energy and carbon dioxide. Thus the amount of radiolabelled substrate taken up by the cells from the growth medium is represented by the radioactivity occurring in the entire cells and the carbon dioxide produced by the fermenter culture.

The lipid fraction was extracted from the cells as described in Chapter 2, Section 2.7 and the total label incorporated was determined as was the label distribution within different lipid classes as outlined in Chapter 2, Section 15. In the course of the extraction, an aqueous phase containing water soluble compounds was produced. The total label in this fraction was also determined. Figs. 6.6, 6.7 and 6.8 illustrate the distribution of label obtained during incubation with ^{14}C glucose, ^{14}C alanine and ^{14}C glutamic acid respectively.

A comparison of the uptake into the different fractions is given in terms of μmoles of radiolabelled compound incorporated calculated from the radioactive counts and specific activities.

These are shown in Figs. 6.7, 6.8, 6.9 and 6.10 for the whole cells, carbon dioxide released, aqueous phase and lipid fraction respectively.

6.2.7 Radiolabelled glucose uptake

There was a steady accumulation of ^{14}C glucose into the A. niger cells throughout the 24h of giant cell formation (Fig. 6.6). The accumulation was particularly marked in the 12-24h period when the radioactive counts rose from 26 x 10^4 dpm to 100 x 10^4 dpm per fermenter culture. The decrease in temperature to 30°C at 24h allowing conidiation to occur, was accompanied by a further acceleration in the rate of label accumulation. This increased rate of accumulation of ^{14}C glucose was maintained to the completion of the microcycle at 48h when the level of ^{14}C glucose present reached 59.8 x 10^4 dpm per fermenter culture.

There was a marked increase in cellular glucose consumption and subsequent release of radiolabelled carbon dioxide from 0 to 36h. The radioactive counts recorded rose from 2 x 10^4 dpm per fermenter culture in the 0 to 12h period, to 15 x 10^4 dpm for the final twelve hours of the microcycle (36-48h). These trends are represented in Fig. 6.6 which illustrates the cumulative radiolabelled carbon dioxide released from the A. niger cells during microcycle conidiation.

The amount of the glucose radioisotope incorporated into the cellular lipid is also illustrated in Fig. 6.6. Only a small amount of radiolabelled glucose is located in the cellular lipid fraction at the 12h stage of the microcycle.

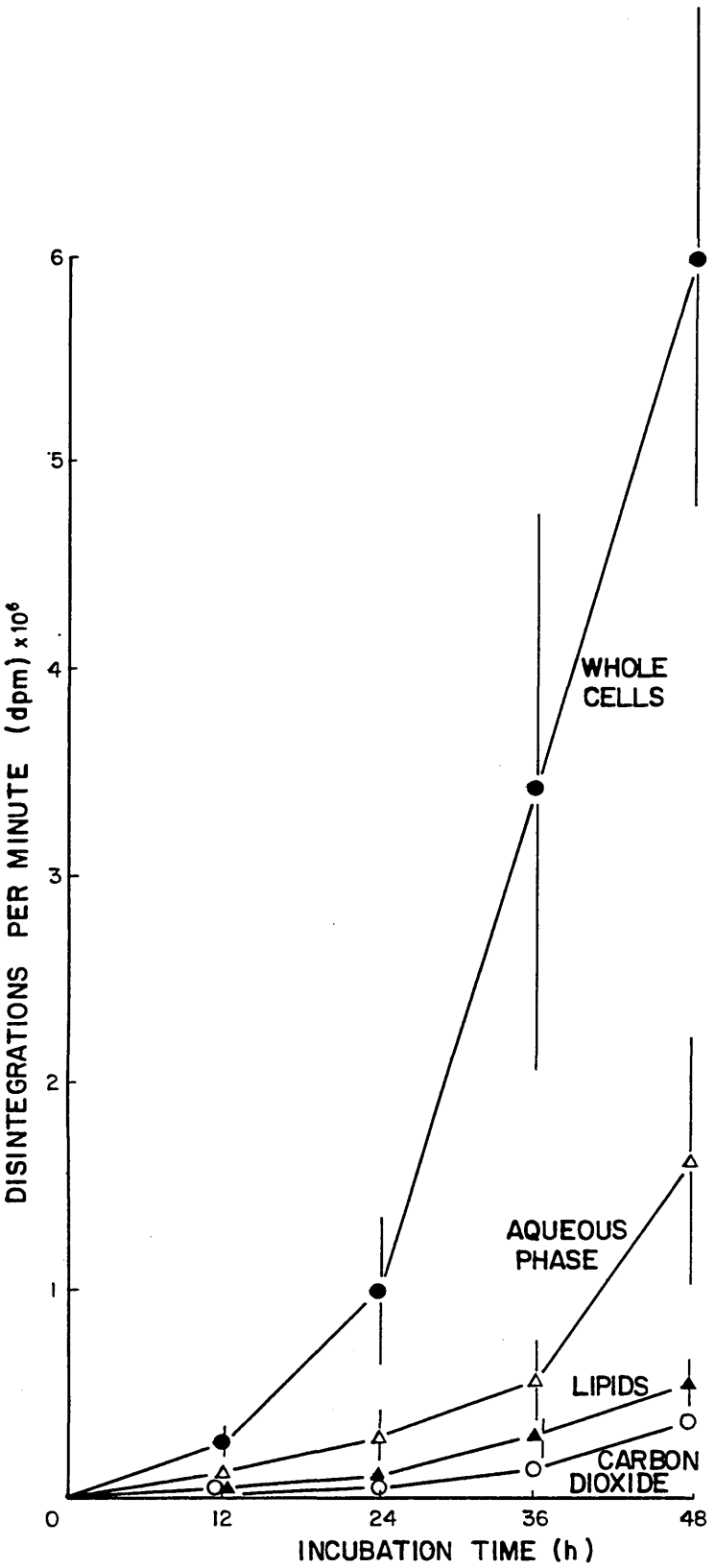


FIG.6.6 THE UPTAKE & FATE OF THE RADIOSOTOPE ^{14}C GLUCOSE DURING MICROCYCLE CONIDIATION IN A. NIGER .

However a dramatic increase in ^{14}C glucose incorporation occurred over the subsequent twelve hours of giant cell formation with radioactive counts rising from 1 x 10^4 dpm per fermenter culture at 12h to 10 x 10^4 dpm at 24h. The rate of ^{14}C glucose incorporation into the lipid fraction increased over the remainder of the microcycle culminating in a radioactive count of 55 x 10^4 dpm per fermenter culture at 48h.

As a result of lipid extraction, an aqueous phase containing non-precipitated water soluble molecules was obtained, and the radioactive counts present were recorded. The counts recorded increased steadily from 12 x 10^4 dpm per fermenter culture at 12h through to 30 x 10^4 dpm at 24h to 57 x 10^4 dpm at 36h. A dramatic increase in ^{14}C glucose incorporation into the aqueous phase occurred between 36 and 48h with a final count of 160 x 10^4 dpm recorded at the end of the microcycle (Fig. 6.6).

6.2.8 Radiolabelled alanine uptake

The amount of ^{14}C alanine incorporated into the A. niger cells increased throughout the 48h microcycle, as illustrated in Fig. 6.7. During the initial twelve hour period of giant cell spherical growth a radioactive count of 58 x 10^4 dpm per fermenter culture was recorded. The counts rose dramatically in the subsequent twelve hour period (12-24h) of giant cell formation reaching 290 x 10^4 dpm by 24h. This high rate of ^{14}C alanine incorporation into the cells continued during the first twelve hour period at 30°C (24-36h).

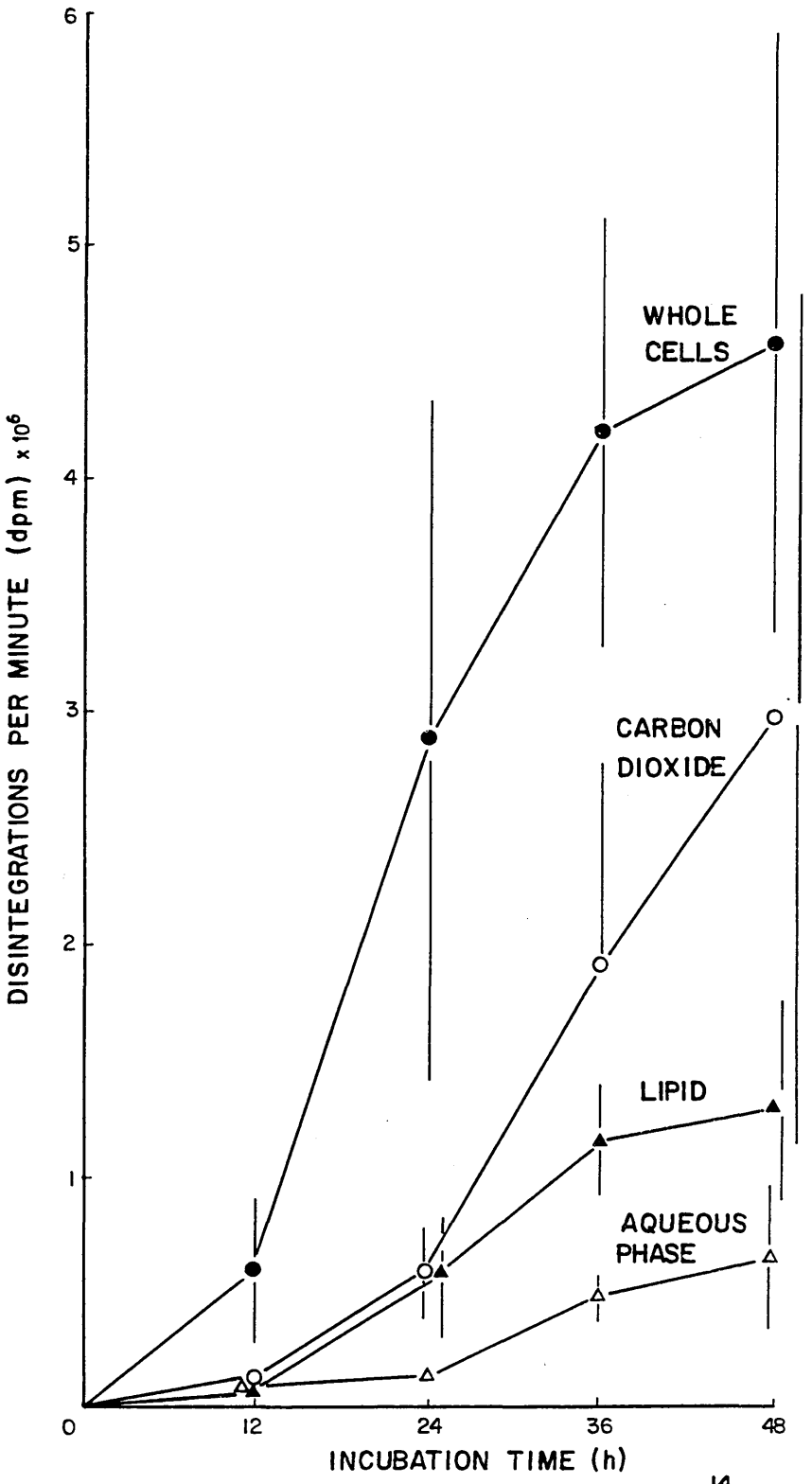


FIG. 6.7 THE FATE OF THE RADIOISOTOPE ^{14}C ALANINE DURING MICROCYCLE CONIDIATION IN A. NIGER.

Although the incorporation of the carbon radioisotope originating from ^{14}C alanine continued over the final twelve hour period of the microcycle, the rate of increase as recorded by radioactive counts, was less dramatic than that observed from 24-36h.

The amount of radiolabelled carbon dioxide emitted by the A. niger fermenter culture increased in each twelve hour period tested from 0-48h of the microcycle. The radiolabelled carbon dioxide emitted in the first twelve hour period of giant cell formation (0-12h) amounted to 8×10^4 dpm per fermenter culture. This radioactive count rose to 49×10^4 dpm per fermenter culture in the final stage of giant cell growth (12-24h) with a dramatic increase to 134×10^4 dpm per fermenter culture recorded for the 24-36h period of conidiophore development. A relatively high radioactive count of 104×10^4 dpm per fermenter culture was maintained during the final twelve hour period of microcycle conidiation to 48h.

Some of the ^{14}C alanine radioisotope supplied in the growth medium was incorporated into the lipids of the A. niger cells. The ^{14}C alanine present in the cells at 12h gave a radioactive count of 6×10^4 dpm per fermenter culture rising sharply to 57×10^4 by 24h. A marked increase in the incorporation of radiolabelled carbon of alanine origin into the cellular lipid was recorded over the conidiophore development period with a radioactive count of 115×10^4 dpm per fermenter culture recorded at 36h. The rate of incorporation of radiolabelled carbon from alanine into cellular lipids decreased over the final twelve hour period of the microcycle with a radioactivity of 134×10^4 dpm per fermenter culture noted at 48h (see Fig. 6.7).

Radiolabelled carbon of alanine origin was incorporated steadily into the aqueous phase of lipid extraction over the twenty-four hour period of giant cell formation. A dramatic increase in the incorporation of ^{14}C from alanine into these water soluble molecules occurred during conidiophore development with radioactive counts rising from 13 x 10^4 dpm at 24h per fermenter culture to 48 x 10^4 dpm at 36h (Fig. 6.7). Incorporation of the ^{14}C from alanine into the molecules of the aqueous phase continued over the 36-48h period, but at a decreased rate.

6.2.9 Radiolabelled glutamic acid uptake

Radiolabelled glutamic acid was taken up rapidly by the giant cells during the first twelve hours of the microcycle with a radioactive count of 40 x 10^4 dpm per fermenter culture recorded at 12h (Fig. 6.8). A much slower rate of uptake of ^{14}C from glutamic acid occurred over the 12-24h period of giant cell formation. However, an increased rate of uptake of radiolabelled carbon from glutamic acid occurred following the decrease in temperature to 30^oC at 24h. During this period of conidiophore development the radioactive count in the cells rose from 45 x 10^4 dpm per fermenter culture at 24h to 84 x 10^4 dpm at 36h. A further increase in the rate of uptake occurred over the final twelve hour period of conidiophore maturation and conidia shedding.

During the initial twelve hour period of the microcycle, very little radiolabelled carbon derived from glutamic acid was emitted as carbon dioxide.

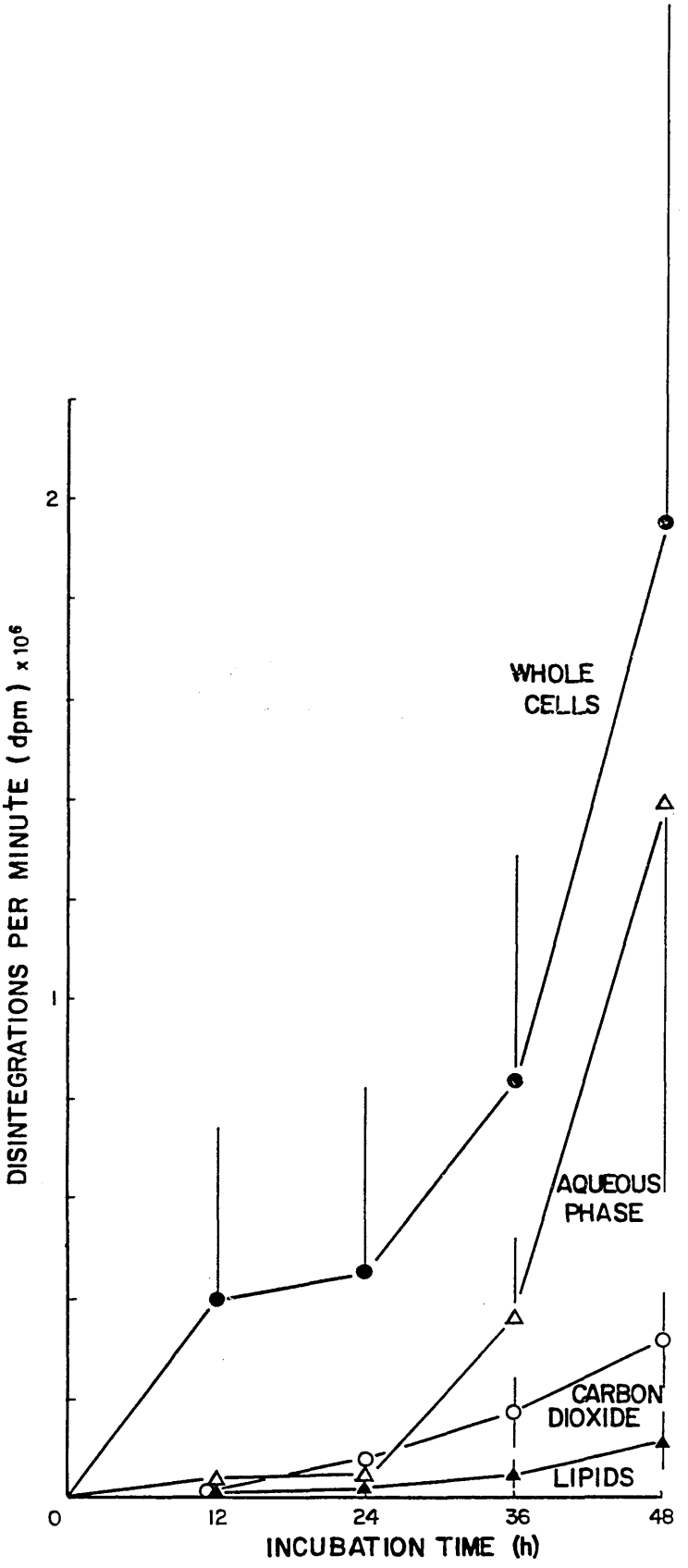


FIG.6.8 THE UPTAKE & FATE OF RADIOISOTOPE ^{14}C GLUTAMIC ACID DURING MICROCYCLE CONIDIATION OF A. NIGER.

However, over the 12-24h period of giant cell formation, an increased emission of radiolabelled carbon dioxide was noted giving a radioactivity of 10 x 10^4 dpm per fermenter culture. A radioactive count of similar magnitude was recorded over the 24-36h period of conidiophore development with a marked increase in radioactivity occurring from 36 to 48h (Fig. 6.8).

Little of the radiolabelled carbon supplied as ^{14}C glutamic acid in the incubation medium, was incorporated into the cellular lipids of the giant cells. Only a radioactivity of 2 x 10^4 dpm per fermenter culture was recorded at 24h, but this increased to 5 x 10^4 dpm at 36h, rising again to 11 x 10^4 dpm by 48h. However, relatively high radioactive counts were obtained for the aqueous phase of lipid extraction, which would contain small, water-soluble molecules. The radiolabelled carbon derived from glutamic acid accounted for a radioactivity of 4 x 10^4 dpm per fermenter culture at 12h. However, little incorporation of the radioisotope into the molecules of the aqueous phase occurred over the subsequent twelve hours. Over the period of conidiophore development, the radioactivity recorded in the aqueous phase rose from 5 x 10^4 dpm per fermenter culture at 24h to 37 x 10^4 dpm at 36h rising again to 140 x 10^4 dpm by 48h.

6.2.10 A comparison of the fate of the three radiolabelled carbon substrates

The radioactivity data recorded as dpm was converted using specific activities, to µmole equivalents of each substrate incorporated into the various samples tested. The results obtained are illustrated in Fig. 6.9 for whole cells, Fig. 6.10 for carbon dioxide emissions, Fig. 6.11 for the aqueous phase of lipid extraction and Fig. 6.12 for cellular lipid. Of the three radioisotope carbon sources provided in the incubation medium, carbon derived from radiolabelled glucose was incorporated into the cells at the highest rate. This rate was particularly high following the decrease in temperature at 24h and reached a maximum of 12, 194 µmole equivalents of glucose per fermenter culture at 48h. Radiolabelled carbon derived from glutamic acid was incorporated at relatively lower rates, although there was a marked increase from 36 to 48h, reaching a maximum of 1856 µmole equivalents of glutamic acid per fermenter culture at 48h. The amount of the radioisotope from ^{14}C alanine taken up by the A. niger cells was very low throughout the microcycle but with a marked increase in uptake from 12 to 24h reaching 184 µmole equivalents of alanine per fermenter culture at 24h.

Radiolabelled carbon of glucose origin was the main carbon source found in the carbon dioxide emitted by the fermenter culture. A marked increase in the rate of ^{14}C carbon dioxide produced from ^{14}C glucose occurred both at 12 and 24h. A cumulative maximum of 747 µmole equivalents of glucose per fermenter culture was observed at 48h. Very little radiolabelled carbon derived from glutamic acid was emitted as carbon dioxide during the first twelve hour period of microcycle conidiation.

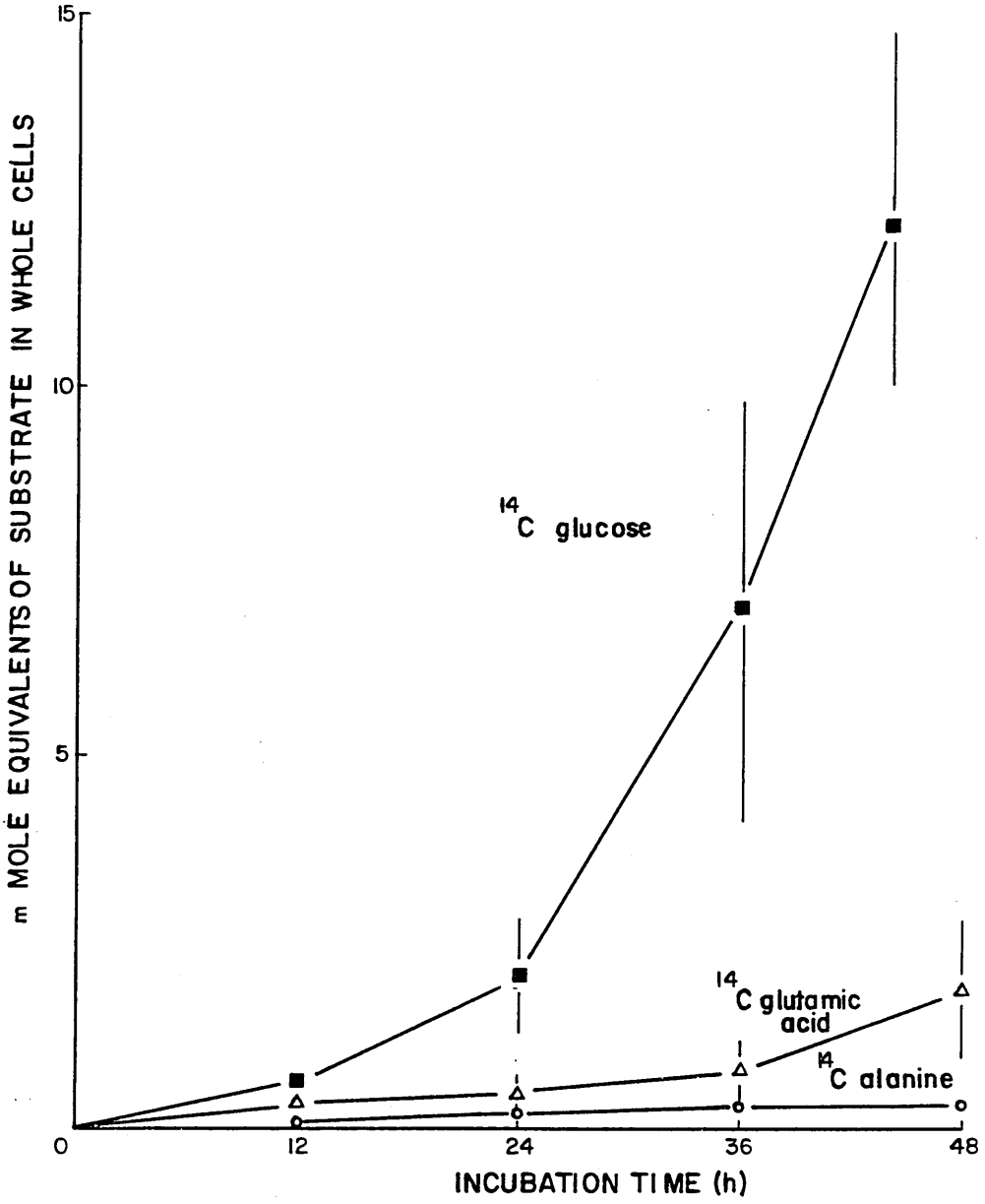


FIG. 6.9 RADIOISOTOPE UPTAKE BY THE CELLS OF A. NIGER THROUGHOUT MICROCYCLE CONIDIATION FROM THE RADIOLABELLED SUBSTRATES SUPPLIED IN THE GROWTH MEDIUM.

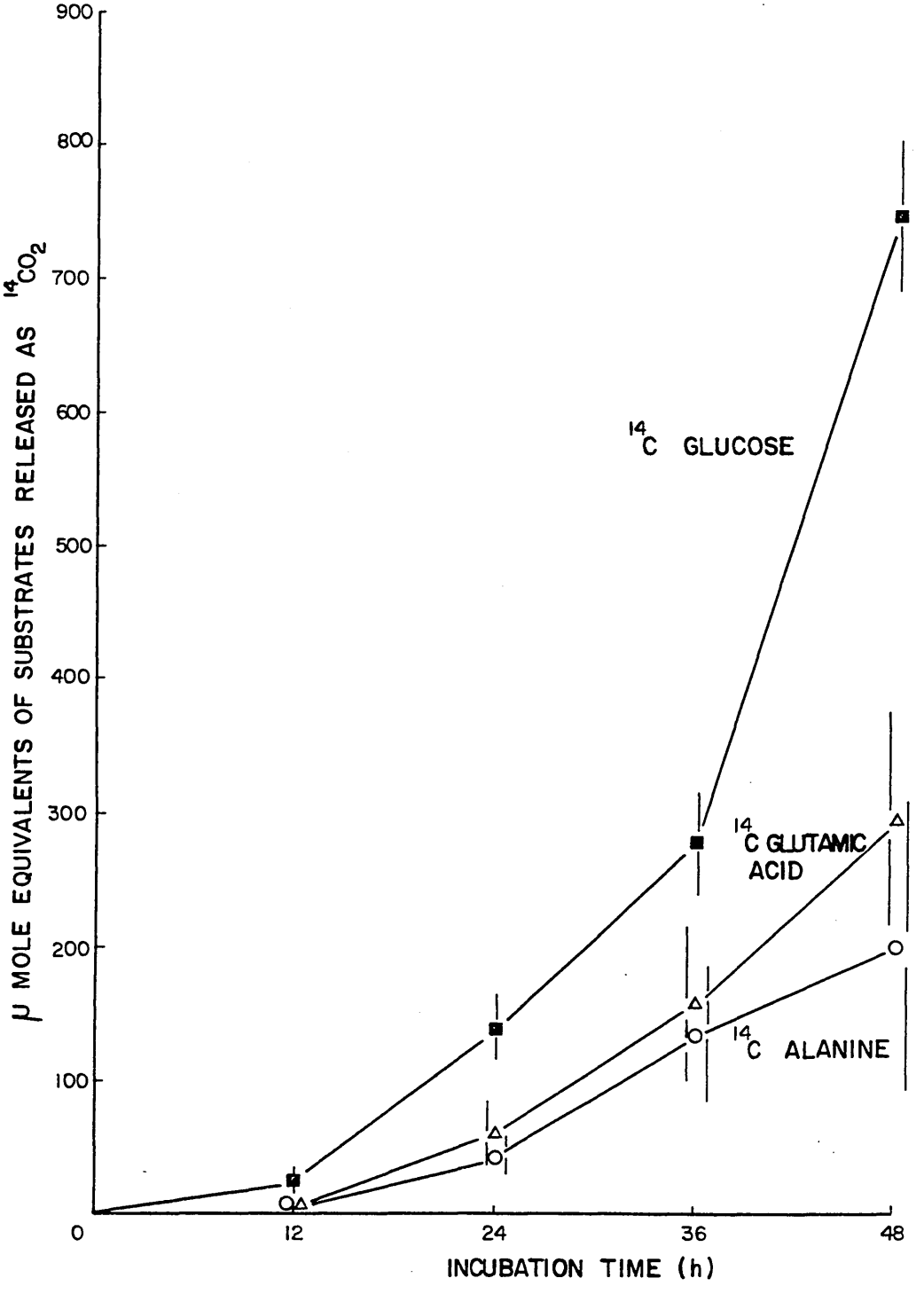


FIG. 6.10 DETERMINATION OF RADIOLABELLED CARBON SUPPLIED IN THE MEDIUM & CONVERTED TO CARBON DIOXIDE DURING MICROCYCLE CONIDIATION IN A. NIGER.

However, this amount increased steadily throughout the cycle reaching a cumulative maximum of 297 µmole equivalents of glutamic acid per fermenter culture at 48h. The amount of radiolabelled carbon of alanine origin was emitted as carbon dioxide in similar proportions to that of the carbon radioisotope derived from glutamic acid. In the 0 to 12h period of giant cell formation, only 6 µmole equivalents of alanine per fermenter culture were emitted as carbon dioxide but this rose to a cumulative total of 203 µmole equivalents by 48h.

The main carbon radioisotope used in the synthesis of cellular lipid during microcycle conidiation in A. niger is ^{14}C glucose. The amounts of the radiolabelled carbon derived from glucose incorporated into lipids increased throughout the cycle. At 12h, 19 µmole equivalents of glucose per fermenter culture had been incorporated into lipid rising to 202 µmoles by 24h. The increase in the rate of incorporation into cellular lipid continued to 36h when 600 µmole equivalents of glucose had been used in lipid synthesis. The increased rate of incorporation continued through the final stages of microcycle conidiation reaching 1126 µmoles of glucose per fermenter culture by 48h.

The radiolabelled carbon derived from both glutamic acid and alanine was incorporated into cellular lipid in much smaller amounts than those recorded for ^{14}C glucose. Only 2 µmole equivalents of glutamic acid per fermenter culture were incorporated into cellular lipid between 0 and 12h. This incorporation rose to 12 µmole equivalents of glutamic acid per fermenter culture by 24h rising to 37 µmole equivalents at 36h.

The greatest amount of incorporation of the carbon radioisotope into lipid occurred between 36 and 48h reaching a maximum of 94 μ mole equivalents of glutamic acid per fermenter culture at 48h. The amount of radiolabelled carbon of alanine origin incorporated into cellular lipid was very low in the 0 to 12h period. However a marked increase in incorporation occurred in the 12 to 24h period with values rising from 5 μmole equivalents of alanine per fermenter culture used in lipid synthesis at 12h to 47 μmole equivalents at 24h. This increased rate of incorporation continued to 36h by which time 92 μmole equivalents of alanine per fermenter culture had been used in lipid synthesis. However in the final twelve hour period of the microcycle only a small amount of the carbon radioisotope from ^{14}C alanine was incorporated into cellular lipid (Fig. 6.12).

A comparison of the uptake of the three sources of radiolabelled carbon into the aqueous phase of lipid extraction is give in Fig. 6.11. It is noted that glucose is used to the greatest extent in the synthesis of the small, water-soluble molecules of the aqueous phase. A maximum of 3346 μmole equivalents of glucose per fermenter culture was incorporated into the aqueous layer by 48h. It is also apparent that the radiolabelled carbon of glutamic acid origin is used in relatively high amounts in the synthesis of molecules found in the aqueous layer of lipid extraction. The incorporation of the carbon radioisotope of glutamic acid was relatively low during giant cell formation to 24h, but increased dramatically during the stages of conidiophore development.

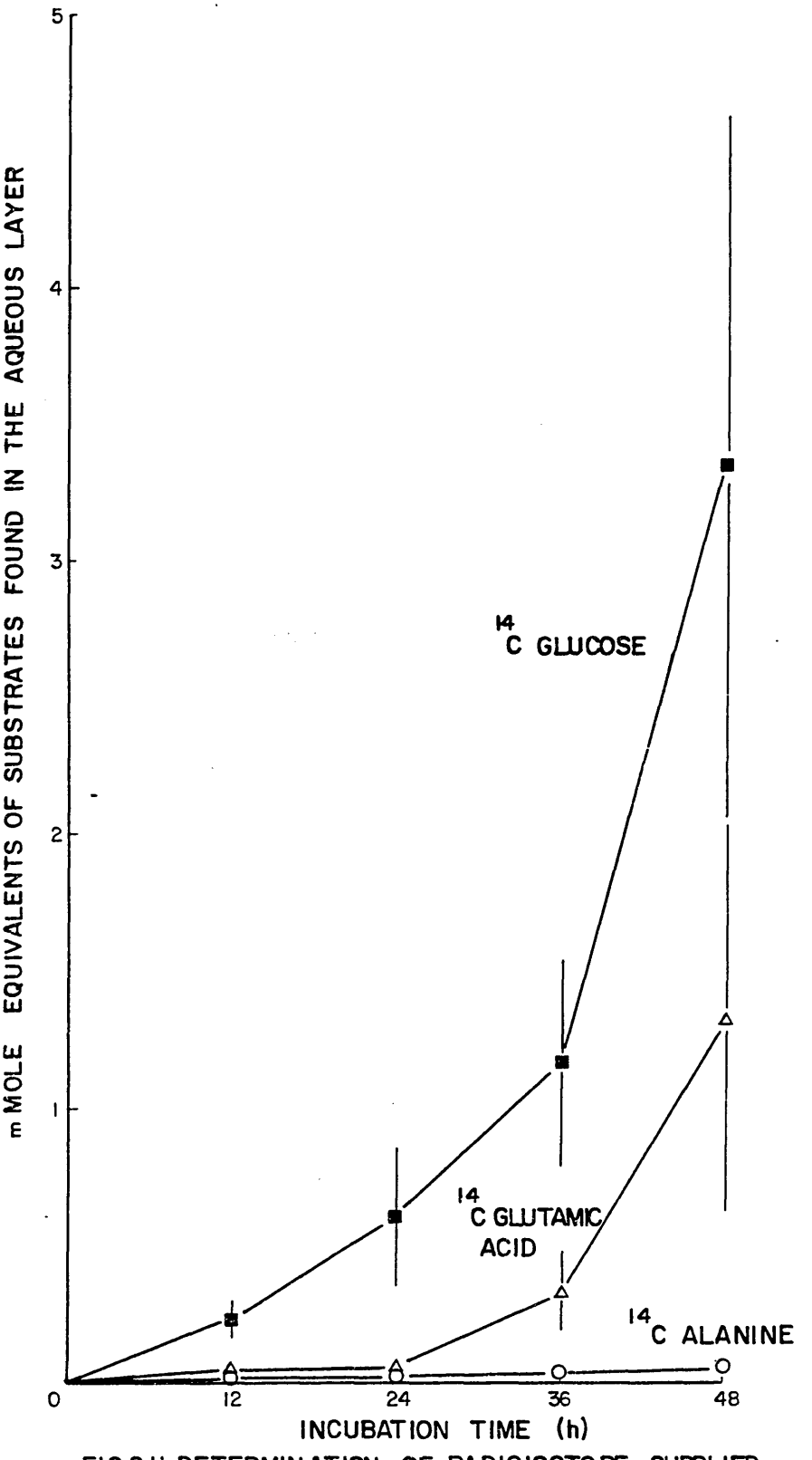


FIG.6.11 DETERMINATION OF RADIOISOTOPE SUPPLIED IN MEDIUM INCORPORATED INTO THE AQUEOUS LAYER DURING LIPID EXTRACTION THROUGHOUT MICROCYCLE CONIDIATION IN A. NIGER.

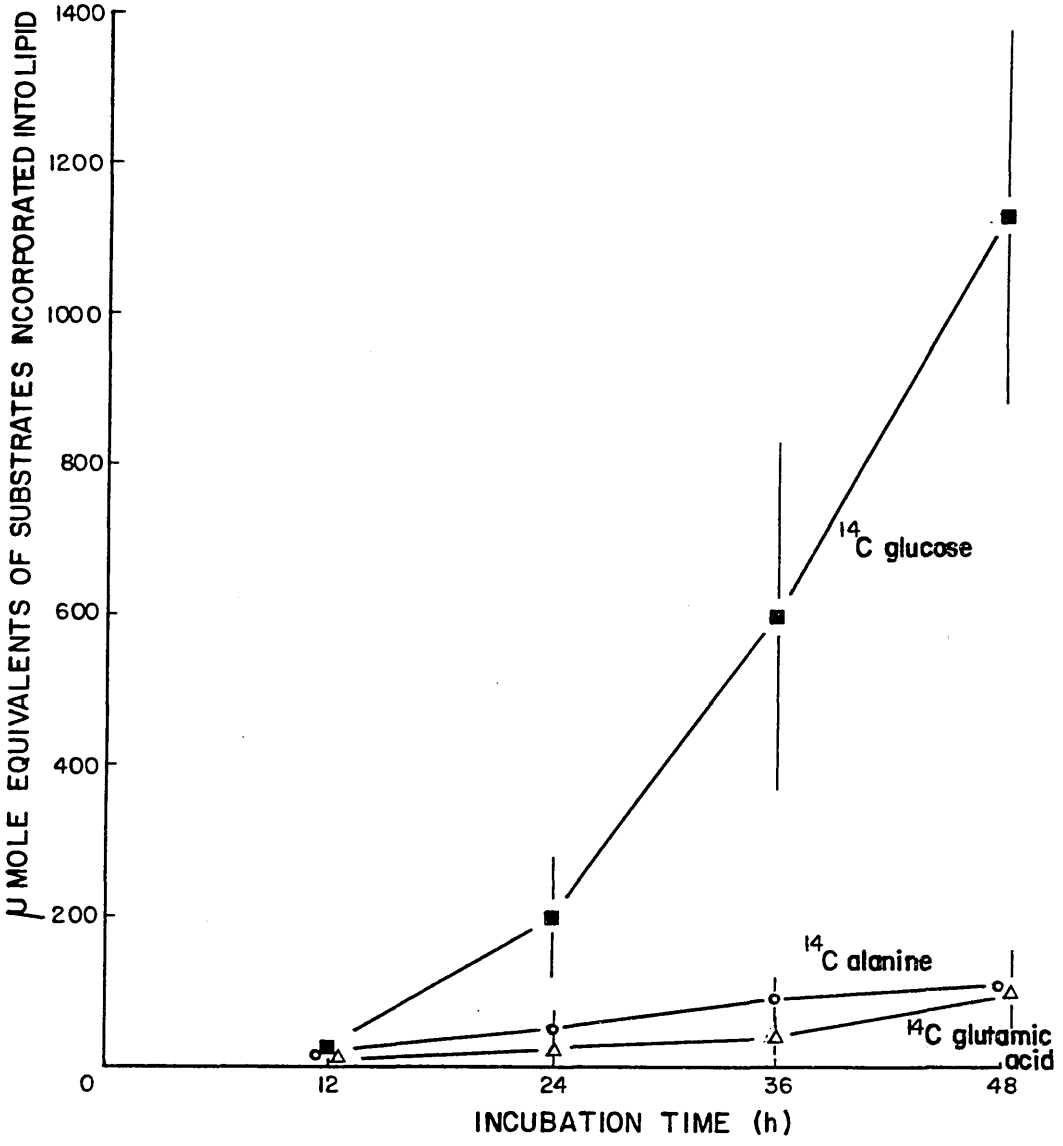


FIG. 6.12 INCORPORATION OF RADIOLABELLED SUBSTRATES, GLUCOSE, ALANINE AND GLUTAMIC ACID INTO THE LIPID OF A.NIGER CELLS UNDERGOING MICROCYCLE CONIDIATION.

The incorporation into the aqueous layer increased from 50 µ mole equivalents of glutamic acid per fermenter culture at 24h, to 336 µmoles at 36h and to 1319 µmoles by 48h. The carbon radioisotope from alanine was incorporated only in very small amounts throughout microcycle conidiation reaching a maximum of 46 µmole equivalents of alanine per fermenter culture by 48h.

6.2.11 The fate of the carbon radioisotopes supplied in the incubation medium expressed as percentage incorporation into various cellular fractions

The cumulative and incremental fates of the carbon radioisotope derived from glucose are expressed as percent incorporation into insoluble material, carbon dioxide emissions, lipid and the aqueous phase of lipid extraction in Table 6.1. The cumulative percentage data illustrate that the majority of the radiolabelled carbon from ^{14}C glucose was used in the synthesis of insoluble materials. A smaller percentage, ranging from 3.93% at 12h to 8.18% at 48h was used in the synthesis of the small, water-soluble molecules found in the aqueous layer of lipid extraction. Some radiolabel was incorporated into lipids (0.32 to 2.75%) with very small amounts emitted as radiolabelled carbon dioxide (0.44 - 1.83%). If the data is presented on an incremental basis (Table 6.1) a similar pattern of incorporation is noted except in the 12 to 24h period. During this period, although only 2975 µmoles of glucose was taken up by the cells per fermenter culture, 6.15% of this was used in lipid synthesis, 12.50% was taken up into the molecules of the aqueous layer and 3.83% emitted as radiolabelled carbon dioxide.

There was a corresponding decrease in radiolabel incorporated into the insoluble material fraction to 77.5% between 12 and 24h.

From the data illustrated in Table 6.2, it was noted that during the 12-24h period there was an increase in the percentage of radioisotope derived from glucose and emitted as carbon dioxide. Only a small amount of the carbon radioisotope from glutamic acid was incorporated into cellular lipid but greater amounts of the isotope, reaching a maximum of 17.2% at 48h were incorporated into the molecules of the aqueous layer. The majority of the radioisotope uptake, however, was incorporated into the insoluble material of the cell.

The carbon radioisotope derived from alanine was also mainly incorporated into the insoluble cellular material with this fraction accounting for 79 - 93% of the uptake in cumulative terms. The amount of radiolabelled carbon of alanine origin emitted as carbon dioxide was relatively low during the 0 to 24h period of giant cell formation, but increased markedly after the temperature decrease to $30^{o}C$ at 24h. The percentage of the radiolabel from ^{14}C alanine taken up by the cells and converted to lipid was comparable to the percentage obtained with ^{14}C glucose (Table 6.1) and was higher than the corresponding ^{14}C glutamic acid percentage (Table 6.2). However, the amount of radiolabelled carbon derived from alanine used in the synthesis of the molecules found in the aqueous layer of lipid extraction was relatively low ranging from 1.3 to 2.8% of the uptake in cumulative terms.

Incubation Time (h)	Cumulative % fate of radiolabelled carbon from glucose				Uptake total (µmoles)
	Insoluble material	CO_2 emissions	Lipid	Aqueous phase	
12	95.3	0.44	0.32	3.93	5950
24	89.4	1.57	2.26	6.79	8925
36	87.3	1.73	3.78	7.26	16030
48	87.2	1.83	2.75	8.18	40897

Incubation Time (h)	Incremental % fate of radiolabelled carbon from glucose				Uptake total (µmoles)
	Insoluble material	CO_2 emissions	Lipid	Aqueous phase	
0-12	95.3	0.44	0.32	3.93	5950
12-24	77.5	3.83	6.15	12.50	2975
24-36	84.6	1.94	5.95	7.85	7105
36-48	87.2	1.89	2.83	11.21	24867

Table 6.1 The cumulative and incremental (12h periods) percentage incorporation of radiolabelled carbon from ^{14}C glucose into the insoluble material, carbon dioxide emissions, lipid and the aqueous layer of lipid extraction per fermenter culture throughout microcycle conidiation.

Incubation Time (h)	Cumulative % fate of radiolabelled carbon from glutamic acid				Uptake total (µmoles)
	Insoluble material	CO_2 emissions	Lipid	Aqueous phase	
12	0	0	0	0	0
24	62.2	17.8	4.9	15.1	331
36	86.9	3.8	1.0	8.2	4092
48	82.4	3.0	1.1	13.4	9817

Incubation Time (h)	Incremental % fate of radiolabelled carbon from glutamic acid				Uptake total (µmoles)
	Insoluble material	CO_2 emissions	Lipid	Aqueous phase	
0-12	0	0	0	0	0
12-24	62.2	16.3	4.1	4.8	331
24-36	89.1	2.6	0.7	7.6	3761
36-48	79.3	2.4	1.1	17.2	5725

Table 6.2 The cumulative and incremental (12h periods) percentage incorporation of radiolabelled carbon from ^{14}C glutamic acid into the insoluble material, carbon dioxide emissions, lipid and the aqueous phase of lipid extraction throughout microcycle conidiation.

Incubation Time (h)	Cumulative % fate of radiolabelled carbon from alanine				Uptake total (μ moles)
	Insoluble material	CO_2 emissions	Lipid	Aqueous phase	
12	93.1	2.5	1.9	2.5	236
24	87.1	5.6	6.0	1.3	786
36	79.9	10.3	7.0	2.8	1313
48	79.0	12.2	6.0	2.8	1661

Incubation Time (h)	Incremental % fate of radiolabelled carbon from alanine				Uptake total (μmoles)
	Insoluble material	CO_2 emissions	Lipid	Aqueous phase	
0-12	93.1	2.5	1.9	2.5	236
12-24	84.6	6.9	7.7	7.8	550
24-36	69.1	17.3	8.6	5.0	527
36-48	75.6	19.5	2.2	2.7	348

Table 6.3 The cumulative and incremental (12h periods) percentage incorporation of radiolabelled carbon from ^{14}C alanine into the insoluble material, carbon dioxide emissions, lipid and the aqueous phase of lipid extraction, throughout microcycle conidiation.

6.2.12 Analysis of the lipid fraction using radioisotope tracers

Further investigations into the fate of the radiolabelled substrates glucose, glutamic acid and alanine with reference to lipid synthesis were carried out by separating the various lipid classes using thin layer chromatography (Chapter 2.9.2). In the case of glutamic acid, the resultant counts in each lipid class were two low (10-500 dpm) in most samples to be reliable representations, although higher counts were obtained in the 48h cells for the phospholipids (polar lipids) and fatty acids. The pattern of incorporation of the radiolabelled carbon supplied as ^{14}C glucose into the various lipid classes is illustrated in Fig. 6.13 and that for ^{14}C alanine in Fig. 6.14.

During the first twelve hours of giant cell formation (0-12h), most of the radiolabelled carbon derived from the glucose molecule is used in the synthesis of polar lipids. In this initial period, 19 µmole equivalents of glucose (see Table 6.1) were converted to lipids and 35.2% of this quantity was used in polar lipid synthesis (see Table 6.4). A relatively small amount of fatty acid, triacylglycerol, sterols and sterol esters were synthesised using the carbon radioisotope from ^{14}C glucose at this stage. The relative percentage of polar lipid present when compared to total lipid, increased up to 24h into the microcycle. A relative accumulation of triacylglycerols occurred over the 12 to 24h period of giant cell formation accompanied by a slight decrease in the sterol ester percentage present and little change in either sterol or fatty acid relative composition.

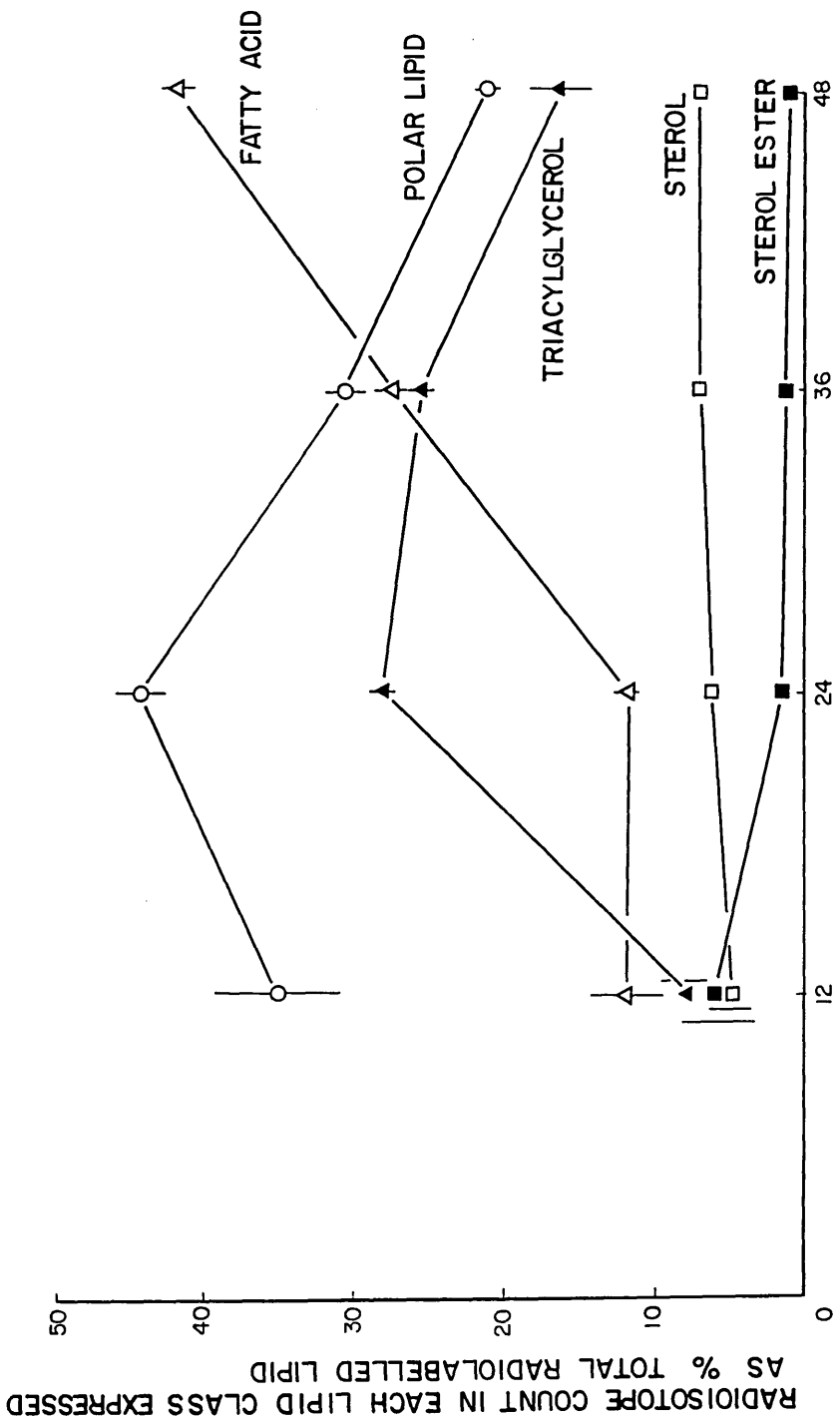


FIG.6.13 THE INCORPORATION OF THE RADIOISOTOPE SUPPLIED AS ^{14}C GLUCOSE INTO THE LIPIDS OF A.NIGER DURING MICROCYCLE CONIDIATION, AS SEPARATED BY TLC.

However, the μmole equivalents of glucose incorporated into fatty acids increased markedly throughout the cycle reaching 472 μmoles by 48h. Similarly the μmole equivalents of glucose incorporated into both sterol and sterol esters increased throughout the cycle (Table 6.1).

The decrease in temperature to 30°C at 24h was accompanied by an increase in the radiolabelled carbon incorporated into free fatty acids. The amount of the radioisotope derived from glucose and used in the synthesis of fatty acids increased relative to total lipid content from 11.9% at 24h, to 41.9% at 48h. Over this period of conidiophore development, the relative amounts of the carbon radioisotope incorporated into both polar lipids and triacylglycerols decreased, but little change occurred in the sterol and sterol ester fractions.

During the preliminary twelve hours of the microcycle, the greatest relative percentage of the radiolabelled carbon from ^{14}C alanine incorporated into the total lipid extract, was located in the polar lipid fraction (Fig. 6.14). The relative percentage of the carbon radioisotope derived from alanine in the polar lipid fraction decreased from 12h to the end of the microcycle. However the μmole equivalents of ^{14}C alanine used in polar lipid synthesis increased from 2.8 μmoles at 12h to 21.1 μmoles at 24h rising to 30.0 μmoles by 36h (Table 6.4). A subsequent decrease was noted to 22.8 μmole equivalents of ^{14}C alanine incorporated into polar lipid at 48h.

Triacylglycerol containing radioisotope carbon derived from alanine was present at a level of 16% at 12h, with an increase to 26% at 24h followed by a slight decrease to 23% at 36h rising again to 29% by 48h.

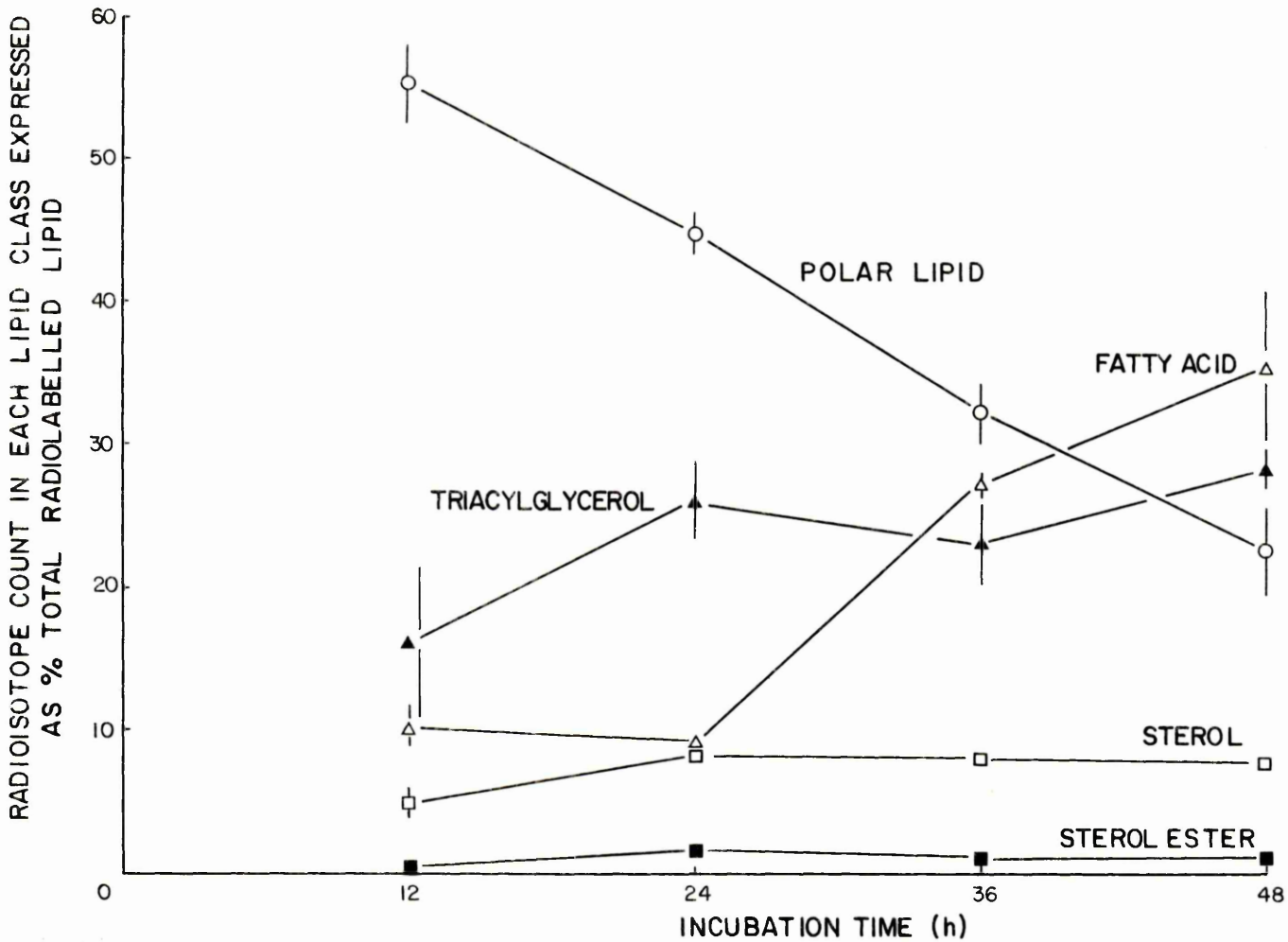


FIG.6.14 THE INCORPORATION OF THE RADIOISOTOPE SUPPLIED AS ^{14}C alanine INTO THE LIPIDS OF A.NIGER DURING MICROCYCLE CONIDIATION, AS SEPARATED BY TLC.

However, only 0.8 µmole equivalents of alanine was used in triacylglycerol in the 0 to 12h period but this amount increased throughout the cycle reaching 28.5 µmoles by 48h. During the preliminary twelve hour period of the microcycle, fatty acid accounted for 10% of the total labelled lipid present. This level dropped to 9% by 24h but increased throughout conidiophore production reaching 35.6% by 48h. Again, the actual µmole equivalents of ^{14}C alanine incorporated into the fatty acids increased throughout the cycle (Table 6.4). The amount of radioisotope from ^{14}C alanine incorporated into the sterol fraction was relatively low throughout the microcycle, with levels of 5% recorded at 12h, 8% at both 24 and 36h and 7.7% at 48h. The actual µmole equivalents of alanine incorporated into sterols was also low, ranging from 0.2 µmoles at 12h to a maximum of 7.7 µmoles at 48h. The levels of radiolabelled carbon from ^{14}C alanine incorporated into sterol esters was very low ranging from 0.3% at 12h to 1.2% at 48h. The µmole equivalent amounts of ^{14}C alanine used in sterol ester synthesis were also very low (Table 6.4).

Incubation time (h)	12		24		36		48	
Lipid class	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Polar lipid	35.2	6.7	44.4	90.1	30.6	183.6	21.4	241.0
Sterol	5.1	1.0	6.3	12.8	7.2	43.2	7.1	79.9
Fatty acid	11.9	2.3	11.9	24.2	27.6	165.6	41.9	471.8
Triacylglycerol	7.8	1.5	28.2	57.2	25.7	154.2	16.4	184.7
Sterol ester	6.0	1.1	1.7	3.5	1.5	9.0	0.9	10.1

(a) Dpm as % total lipid count

(b) µmoles equivalent glucose

Incubation time (h)	12		24		36		48	
Lipid class	(c)	(d)	(c)	(d)	(c)	(d)	(c)	(d)
Polar lipid	55.3	2.8	44.8	21.1	32.3	30.0	22.8	22.8
Sterol	4.9	0.2	8.1	3.8	8.1	7.5	7.7	7.7
Fatty acid	10.4	0.5	8.7	4.1	27.5	25.6	35.6	35.6
Triacylglycerol	16.1	0.8	26.0	12.2	23.1	21.5	28.5	28.5
Sterol ester	0.3	0.002	1.6	0.8	1.0	0.9	1.2	1.2

(c) Dpm as % total lipid count

(d) µmoles equivalent alanine

Table 6.4 The incorporation of the carbon radioisotope supplied as ^{14}C glucose and ^{14}C alanine into the various lipid classes, expressed as a percentage of total lipid present and as µmole substrate used.

6.4 DISCUSSION

Glucose is the main carbon source supplied during the present microcycle conidiation study in A. niger. The radiotracer data show that 92.1% of the 10g/l (44,450 μ moles) in the original growth medium is utilised by the end of the 48h period (Fig. 6.3). Confirmation of the extent of glucose uptake was obtained by chemical analyses with 91.1% of the supplied substrate taken up from the medium by the final stage of microcycle conidiation. Glucose will thus give rise quantitatively to the major part of all cellular constituents.

Glutamic acid is the second main carbon source with 5g/l supplied in the incubation medium. The only other carbon source supplied with glucose and glutamic acid in the growth medium was alanine which was initially at a concentration of 0.2g/l. Little glutamic acid is taken up by the A. niger cells over the twenty-four hour period of giant cell formation, as is evident from Fig. 6.3. However, there was a marked increase in glutamic acid uptake by the cells over the period of conidiophore production and development. These trends are reflected in Fig. 6.5 which illustrates the fluctuations in the levels of ninhydrin reactive material over the microcycle. All substances possessing a free NH_2 group would be quantitated by this method, and glutamic acid would be expected to be one of the major classes of reactive material. Although alanine was taken up by the cells throughout the 48h period of microcycle conidiation (see Fig. 6.3), this uptake would be masked by the presence of glutamic acid during the quantitation of the ninhydrin reactive material.

The decrease in radiolabelled substrates present in the growth medium during microcycle conidiation is due to the intake of the substrates by the developing giant cell. However, the possibility must be considered that some radiolabelled carbon may be taken into the A. niger cells and used in the synthesis of extracellular material which may be secreted back into the growth medium. In the present study, these would not have been separately identified as distinct from the added radiolabelled substrates.

The ^{14}C glucose, ^{14}C glutamic acid and ^{14}C alanine radioisotopes were traced into the whole cells, cellular lipid, the aqueous layer of lipid extraction and carbon dioxide emissions (Figs. 6.6, 6.7 and 6.8). Some difficulty was experienced in producing a homogeneous sample of the entire contents of the A. niger cells undergoing microcycle conidiation, even with the use of NCS tissue solubilizer. This resulted in relatively low counts for the whole cells (Fig. 6.9) when compared to the recorded uptake of radiolabelled substrates from the medium (Fig. 6.3). However, the whole cell radioactivity can be calculated from the uptake measurements and the carbon dioxide loss. These data are shown in Fig. 6.16 and are considered to be more reliable estimates of the whole cell radioisotope content. A comparison of the dry weight values obtained by direct measurement (Fig. 4.3) with those calculated from the radiolabelled data is given in Fig. 6.17.

The majority of the radiolabel supplied in all three carbon sources is incorporated into the insoluble material of the A. niger cells. This material would include insoluble proteins, cell wall constituents, covalently bound lipids and also nucleic acids and proteins precipitated out by the trichloroacetic acid added to the harvested cells.

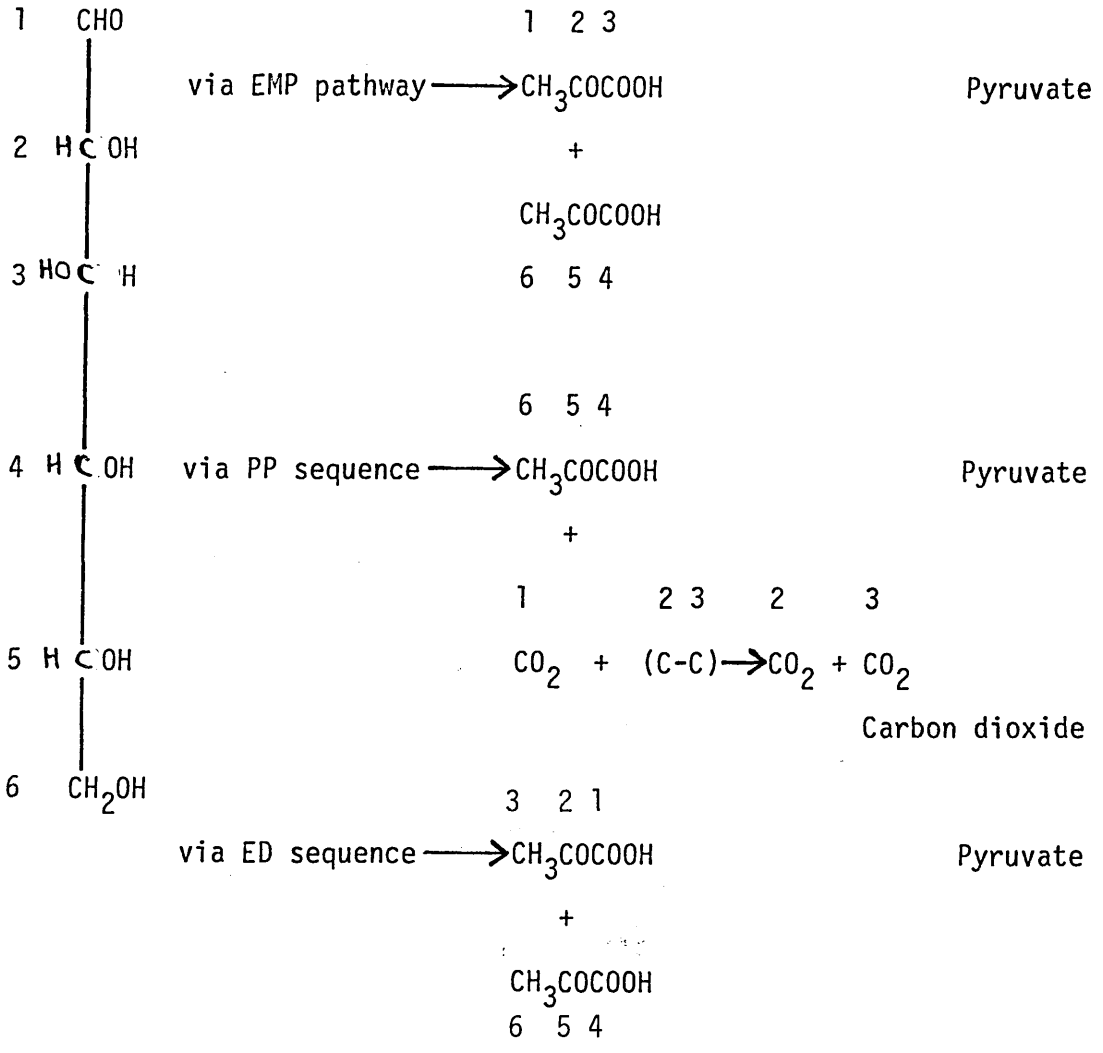


Fig. 6.15 The breakdown of glucose by the three glycolysis pathways.

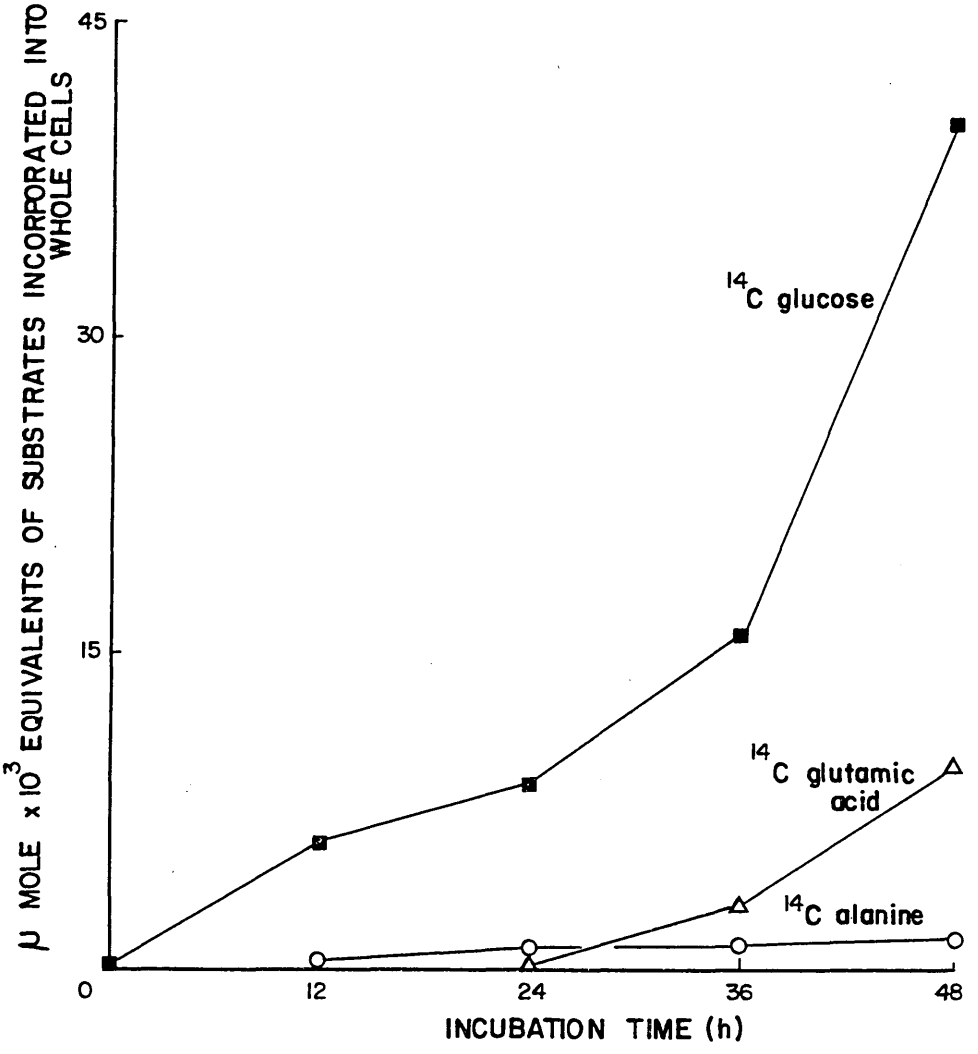


FIG.6.16 RADIOISOTOPE UPTAKE BY CELLS OF A.NIGER THROUGHOUT MICROCYCLE CONIDIATION, FROM THE RADIOLABELLED SUBSTRATES SUPPLIED IN THE GROWTH MEDIUM. THE DATA WAS CALCULATED BY DIFFERENCE FROM THE RADIOISOTOPE COUNTS IN THE MEDIUM AND CARBON DIOXIDE.

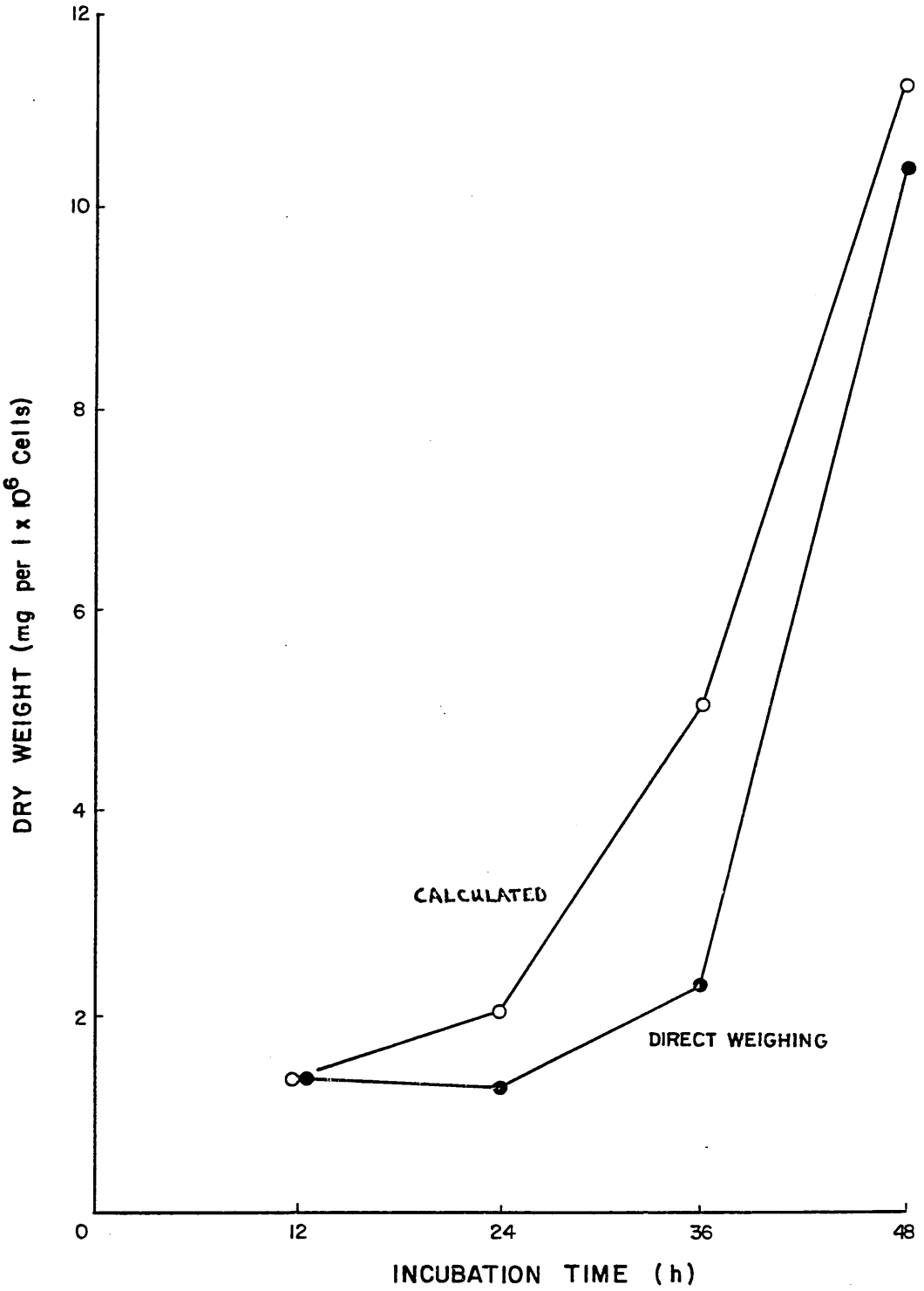


FIG.6.17 THE COMPARISON OF DRY WEIGHT VALUES OBTAINED BY DIRECT WEIGHING OF CELLULAR MATERIAL WITH THE VALUES CALCULATED FROM RADIOISOTOPE ANALYSIS INDICATING THE AMOUNT OF CARBON CONTAINING COMPOUNDS SYNTHESISED.

During the first twelve hours of giant cell formation 95.3% of the carbon radioisotope supplied as ^{14}C glucose is utilised in the production of insoluble cellular material. A thickening of the giant cell wall occurred during spherical growth and the insoluble cellular material would be expected to contain cell wall constituents. Some of the carbon radioisotope supplied is also used in the production of the small, water-soluble, non-precipitated molecules of the aqueous phase of lipid extraction. However very little lipid production occurred in this 0-12h period and energy demands by the cells was minimal as only 0.44% of the carbon radioisotope was emitted as radiolabelled carbon dioxide.

Over the second twelve hour period of giant cell formation the uptake of glucose from the growth medium was only 2975 µmoles as compared to 5950 µmoles in the 0 to 12h period. Of these 2975 µmoles, proportionally less (77.5%) of the radiolabelled carbon was incorporated into insoluble cellular material. However, there was a relatively higher incorporation into the water soluble molecules of the aqueous phase, lipid and carbon dioxide emissions. This suggests greater metabolic activity and the build-up of lipid reserves by the cells at this stage. In the 24 to 36h period of conidiophore production and development, 7105 µmoles of radiolabelled glucose was taken up by the cells from the incubation medium. The vast majority of the radiolabelled carbon was incorporated into insoluble cellular material needed for the developing conidiophore structure, with smaller amounts used in the production of lipid, small water-soluble molecules of the aqueous phase and carbon dioxide. During the final twelve hour period of microcycle conidiation, the glucose taken up by the cell increased to 24,867 µmoles.

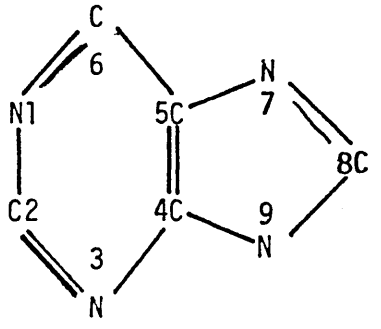
Again, the majority of the carbon radioisotope was incorporated into the insoluble cellular material. A relatively high proportion (11.21%) was incorporated into the water soluble molecules of the aqueous phase with smaller amounts used in lipid production and the emission of carbon dioxide.

Each molecule of glucose yields two molecules of pyruvate when broken down via the EMP pathway, whereas only one molecule of pyruvate plus carbon dioxide and a two carbon compound when oxidised via the PP pathway (see Fig. 6.15). In the normal, mycelial growth form of A. niger, a higher activity of the PP pathway prior to sporulation has been observed (Carter & Bull, 1969; Ng, Smith & Anderson, 1972). However, in the presence of glutamic acid, which itself induces sporulation in A. niger, the EMP pathway seems to be more active (Smith, Valenzuela-Perez & Ng, 1971). Fluctuations in the concentrations of enzymes of the EMP and PP pathways were noted well in advance of any morphological changes.

The carbon skeleton of glucose can be used in the production of many other compounds in the fungal cell, as illustrated in Fig. 6.1. The intermediates of glycolysis are used in the synthesis of phospholipids, pentoses (precursors of ribose phosphate and deoxyribose phosphate, RNA and DNA components respectively) and triacylglycerols. The end product of glycolysis is pyruvate which can be converted to acetyl CoA with the loss of a molecule of carbon dioxide. Acetyl CoA is the precursor of fatty acids and sterols, and can also enter the tricarboxylic acid (TCA) cycle. The esterification of fatty acids with glycerol results in triacylglycerols, and with sterols, sterol esters are produced.

Some amino acids are produced from the intermediates of glycolysis and will therefore utilise the carbon skeleton of glucose. Glycerate-3-phosphate is a precursor of serine and hence of glycine and cysteine; phosphoenolpyruvate is the precursor of the aromatic amino acids, phenylalanine, tyrosine and tryptophan. Others use the intermediates of the TCA cycle as precursors and can therefore utilise any of the three carbon substrates provided in the growth medium.

The glucose carbon skeleton may also be involved in the production of nucleic acids which are composed of bases and ribose phosphates or deoxyribose phosphates. The ribose phosphates and the deoxyribose phosphates are formed from the pentoses which are derived from glucose-6-phosphate. The major bases of nucleic acids are the pyrimidines viz. cystosine, uracil and thymine, and the purines, adenine and guanine. The pyrimidines are formed from aspartate which itself is produced from oxaloacetic acid, a four carbon acid of the TCA cycle. Thus the pyrimidines may arise from any of the three carbon substrates, glucose, glutamic acid and alanine supplied in the microcycle conidiation growth medium. A large number of precursors are used piecemeal to produce purines, the end result being:-



The N-9 arises from glutamine, C-4, C-5 and N-7 from glycine and C-8 from a one-carbon unit carried by the co-enzyme, tetrahydrofolate. Then N-3 is added from glutamine, C-6 from carbon dioxide, N-1 from aspartate and finally C-2 from a tetrahydrofolate derivative. Thus two of the carbon atoms arise indirectly from glucose through the formation of glycine from glycerate-3-phosphate. Although none of the carbon atoms of purines derive from glutamic acid, one nitrogen atom is supplied directly from glutamine and the other two are probably supplied indirectly as both glycine and aspartate are usually produced by transamination with glutamine donating the nitrogen group.

As illustrated in Table 6.2, glutamic acid is not taken up and utilised by the A. niger cells during the first twelve hours of spherical growth. During the second twelve hour period of giant cell formation, only 331 μ moles of ^{14}C glutamic acid are taken up by the cells. The majority of this (62.2%) is used in the production of insoluble cellular material, with small amounts used in the production of small, water soluble molecules and lipids. However, 16.3% of the label supplied as ^{14}C glutamic acid was emitted as radiolabelled carbon dioxide.

A pronounced increase in ^{14}C glutamic acid uptake by the cells occurred at the onset of conidiation and continued throughout the period of conidiophore development. The radiolabelled carbon atoms from ^{14}C glutamic acid are mainly incorporated into the insoluble cellular material although they also figure prominently in the molecules of the "aqueous phase" during conidiation.

The presence of glutamic acid is essential for maximum conidiation in A. niger giant cells (Anderson & Smith, 1971, a, b) and it is probable that the presence of glutamate masks the high reductive biosynthetic needs of sporulation. As previously stated, the pentose phosphate pathway of glycolysis is more prominent during sporulation of A. niger, but in the presence of glutamic acid, the EMP pathway is the more active.

Glutamic acid can be deaminated or transaminated to form 2-oxo-glutaric acid which is an intermediate of the TCA cycle. The 2-oxo-glutaric acid may be converted to malic acid via the TCA and hence to pyruvate which can be converted to acetyl CoA. The carbon skeleton of glutamic acid may be used in the formation of other amino acids formed from intermediates of the Krebs cycle. This may explain the increased amounts of radiolabelled carbon atoms of ^{14}C glutamic acid origin found in the aqueous phase of low molecular weight, water soluble molecules during conidiation.

Glutamic acid is an important amino acid as many others derive their α-amino nitrogen from it. In such reactions the amino group is transferred from glutamic acid to other amino acids by transamination. Much of the nitrogen content of the purine bases of DNA and RNA is of glutamate origin. It is therefore apparent that glutamic acid is actively involved in metabolic processes in the A. niger cells undergoing conidiation.

Alanine is supplied in much smaller quantities than glutamic acid in the incubation medium. Over the first twelve hours of giant cell growth, 236 μ moles of ^{14}C alanine were taken up by the cells. The majority of this was used in the production of insoluble cellular material with very small amounts incorporated into lipid, molecules of the aqueous phase and carbon dioxide.

During the second twelve hour period of spherical growth, 550 μ moles ^{14}C alanine was taken up from the growth medium. Although the majority of this was utilised in the production of insoluble cellular material, there was an increased output of radiolabelled carbon dioxide, lipid and the water soluble, low molecular weight compounds of the aqueous phase. A similar amount of ^{14}C alanine uptake from the medium (527 μmoles) was recorded for the 24 to 36h period of conidiophore production. However, the amount incorporated into the insoluble cellular material was lower (69.1%), but with an increase in radiolabelled carbon dioxide emissions to 17.3%. This suggests that alanine figures prominently in the catabolic process of energy production during conidiophore development. The relatively high level of radiolabelled carbon dioxide emissions originating from ^{14}C alanine continued throughout the 36 to 48h period.

Alanine can be readily converted to pyruvate, with the liberation of ammonia. Pyruvate, the end product of glycolysis, can be converted to acetyl CoA which may enter the TCA cycle. The intermediates of the TCA cycle are used in the formation of various cellular constituents, or they can be completely metabolized to produce energy.

Previous studies using radioisotopes have revealed that the highest rates of RNA and protein synthesis occur during the first 20h of giant cell formation (Duncan et al., 1978). This being the case, the data in Figs. 6.3 and 6.9 would suggest glucose as an important precursor for these compounds. Some amino acids used in protein synthesis are derived from the intermediates of glycolysis, e.g. glycerate-3-phosphate is a precursor of serine, glycine and cysteine.

Hence any label found in these amino acids must originate in the radioisotope of glucose. Other amino acids are derived from pyruvate, phospho-enolpyruvate and intermediates of the TCA cycle such as 2-oxoglutarate and oxaloacetate. As illustrated in Fig. 6.1, the radiolabel from any of the three substrates supplied could be used in the synthesis of these precursors of amino acid synthesis. The carbon skeleton of glucose may be involved in the synthesis of all amino acids, with a contribution from alanine and glutamic acid.

The oxidation of glucose can also yield pentoses from which ribose phosphate and deoxyribose phosphate are formed. These sugar phosphates combine with nucleic acids to form DNA and RNA. The nucleic acids are synthesised from purines and pyrimidines. Pyrimidines are formed from aspartate which itself it derived from a C4 intermediate of the TCA cycle, which can originate from any of the three radiolabelled substrates, glucose, glutamic acid or alanine. A large number of precursors are used piecemeal to produce purine but two of the carbon atoms of the ring structure arise from glycine which can itself be produced from glucose. Of the 3 substrates used glucose is of paramount importance in DNA, RNA and protein synthesis, and an increase in the incorporation of radiolabel of glucose origin may, as one possibility, indicate greater activity in the manufacture of these products.

Some of the radiolabelled glucose used in the radio tracer analyses was completely metabolized to yield radiolabelled carbon dioxide and providing the cell with an energy supply. However a far greater quantity of ^{14}C glucose is used in the synthesis of new cellular material as illustrated in the whole cell radioisotope count (see Fig. 6.9) and in Table 6.1.

Included in the whole cell count would be the radiolabel incorporated into the lipid components of the A. niger cells. From 0 to 12h, the lipid fraction amounts to 0.32% of the total new cellular material synthesized using the carbon skeleton of the ^{14}C glucose supplement. The proportion of lipid synthesized by the cell increased to 6.15% of the total cellular material at 24h, dropping to 5.95% at 36h and again at 48h to 2.83% (see Table 6.1). Thus the lipid fraction accounts for a maximum of 6.15% of new cellular material synthesized during microcycle conidiation and this maximum occurs just prior to the onset of conidiation. The low level of lipid synthesis by the cells is consistent with the results of the total lipid analyses (Chapter 4) and a maximum synthesis between 12 and 24h is in agreement with the cell wall lipid results of Deans, 1978. The total lipid content of the giant cells increased at 24h, but since the radioactivity present was only measured at 12h intervals, as opposed to 4h for total lipid, the exact time of greater increase of radiolabel incorporation cannot be pinpointed.

The majority of the ^{14}C glucose taken up by the cell is used in the production of cellular materials other than lipid. The carbon skeleton of glucose may be used in the production of structural polysaccharides including the cell wall chitins, glucans and the carbohydrate storage material, glycogen. A relatively high amount of protein is present in the giant cells of A. niger, as compared to the lipid content. For example at 12h, 7.9 µg of lipid is present per 1×10^6 cells whereas a similar concentration of cells contains 22.9 µg protein. At 24h, the concentration rises to 33.9 µg of lipid per 1×10^6 cells compared to 41.3 µg of protein; and at 48h, 69.7 µg lipid is present with 209.0 µg protein (see Figs. 4.4 and 4.5).

The second twelve hour period of the microcycle up to 24h is a time of continued spherical growth of the giant cell. The lipid formed at this stage may be used for the accumulation of energy reserves in the form of lipid globules. Another possible fate of the synthesized lipid is in the formation of the thickening cell wall which accompanies spherical giant cell growth. A dramatic increase in cell wall lipid content has been previously reported for A. niger giant cells in the 20 - 30h period of microcycle conidiation (Deans, 1978). There will also be a demand for de novo lipid synthesis in the formation of cellular membranes. An increase in nuclear number from six to fourteen has been previously demonstrated (Yahya, 1979; Smith et al., 1981a) and a greater demand for lipid in the formation of nuclear membranes would be expected.

In Chapter 4, it was postulated that the decrease in total cell lipid noted in the 24 - 32h period was due to the mobilisation of the lipid energy reserves for conidiation. It has been stated that cell wall lipids may represent a convenient storage system and a decrease in their quantities has also been noted after the onset of conidiation (Deans, 1978). However, during the development of conidia an increase in both cell wall lipid (Deans, 1978) and the total cell lipid has been demonstrated (Fig. 4.5). Lipid would be expected to be in demand for plasma membrane formation and for the membranes of mitochondria, endoplasmic reticulum, nuclei etc. Lipid might also be required in the build-up of energy reserves for the new conidia.

The glutamic acid and alanine substrates make a less significant contribution to the formation of cell lipids when compared to glucose (see Fig. 6.12). The amount of ^{14}C of glutamic acid origin involved in lipid synthesis increases gradually over the microcycle with most incorporation occurring in the 36 - 48h period, although the maximum percentage contribution to lipid occurred in the 12 to 24h (Table 6.2). This, however represents only a very small amount of synthesis of lipid from glutamate. Very little of alanine's carbon skeleton is involved in lipid production during the 0 - 12h and 36 - 48h microcycle periods. However greater amounts of ^{14}C derived from alanine is used in lipid synthesis in the 12 - 24h period of giant cell formation and in the 24 - 36h period of conidiophore development (Table 6.3). The conversion of glutamic acid to pyruvate is not as simple as the formation of pyruvate from alanine and this may explain the greater impact of ^{14}C of alanine origin on lipid production.

When the data obtained in Fig. 6.12 is represented in terms of the raw counts i.e. disintegrations per minute (dpm), the radiolabelled carbon of alanine origin figures most prominently in cellular lipid as illustrated in Figs. 6.6, 6.7 and 6.8. This indicates that although alanine is present in only small amounts (0.2g/l) in the growth medium, it is metabolically in great demand by the giant cell. A larger proportion of the ^{14}C from the alanine molecule must be used in lipid synthesis, whereas fewer radiolabelled carbons from glucose and glutamic acid may be incorporated into lipid.

Other workers have noted that a supply of organic nitrogen such as glutamate enhances lipid production in certain yeasts when compared to lipid synthesis in the presence of a NH_4^+ salt (Woodbine, 1959; Evans and Ratledge, 1984a). The metabolism in Rhodosporidium toruloides which showed this effect, was significantly different in cells grown on glutamate to those grown on NH_4^+. Excretion of citric acid was pronounced in the glutamate grown cultures, indicating that the conversion of glutamate to citrate was stimulated (Evans and Ratledge, 1984a). Studies with R. toruloides indicate that the intercellular concentration of NH_4^+ is probably of considerable regulatory significance during the initial stages of lipid biosynthesis. An increase in the NH_4^+ concentration prior to the accumulation of citrate and lipid suggests that NH_4^+ must be present at a significant concentration before lipid production is promoted (Evans and Ratledge, 1984b). Accumulation and regulatory significance of NH_4^+ has been reported in a citric acid producing species of A. niger which could accumulate a very high NH_4^+ concentration throughout growth (Rohr and Kubicek, 1981).

Of the glutamate carbon taken into cells of the yeast, R. toruloides CBS14, 27% was recovered in the lipid fraction indicating that not only was glutamate acting as a source of NH_4^+, but that the resultant α-oxoglutarate was being actively metabolized by the cells. However, only a proportion of the glutamate carbon atoms were incorporated into lipid and it was apparent that the increase in lipid produced must be due to an increased metabolic rate arising indirectly from glutamate being used as a nitrogen source, rather than glutamate acting as a direct precursor of the lipid (Evans and Ratledge, 1984a).

During the first 24h of microcycle conidiation, when giant cell formation occurs, the major class of lipids found containing radiolabel of glucose origin are the polar lipids (see Fig. 6.13). This is a period of elevated incubation temperatures (41^{o}C for 11h, followed by 44^{o}C for the next 13h) and it is known that phospholipids, the main group found within the polar lipid class, respond to external stimuli and depending on cell requirements, their structure is modified to help cell survival under changed conditions. Catabolism and turnover of phospholipids has been suggested to be essential for membrane adaption of environmental changes (Raetz, 1978).

Using labelled phosphate as a precursor, phospholipid metabolism in Mycobacterium smegmatis was shown to be influenced by its growth temperature (Taneja and Khuller, 1981). The rate of phospholipid synthesis and degradation was lower in cells grown at 27^{o}C as compared to those grown at 37^{o}C. From Figs. 6.13 and 6.14, it can be seen that the highest relative synthesis of phospholipids (polar lipids) occurs during the first 24h of A. niger microcycle conidiation at the elevated growth temperatures. At the lower temperature of 30^{o}C, from 24 - 48h a decrease in the phospholipid content as a relative percentage of total lipid was noted. However, direct comparisons are difficult here as the lower temperature induces conidiation in the A. niger giant cells, and the temperature effect on phospholipids may be masked by the physiological changes brought about by the onset of conidiation.

The precursors of phospholipids are glycerate-3-phosphate which arises from the phosphorylation of glucose, and fatty acids which are formed from acetyl CoA which is readily produced from pyruvate,

the end product of glycolysis. Pyruvate is also readily produced from alanine, with the liberation of ammonia. The long chained fatty acids can be activated by forming their coenzyme A esters. The activated fatty acids, catalysed by an acyl transferase can esterify 3-sn-glycerophosphate to form 1, 2-diacyl-3-sn-glycerophosphate or phosphatidic acid. Thus three of the carbon atoms of the phosphatidic acid must arise from glucose, the rest of the molecule produced from fatty acids could be produced from glucose, alanine and less readily from glutamic acid via the TCA cycle. Phosphatidic is activated by cytidine triphosphate (CTP) to form cytidine diphosphate-diacylglycerol. This can now react with serine to yield phosphatidyl serine from which phosphatidyl ethanolamine is formed by decarboxylation. Serine is formed from glycerol-3-phosphate and hence from glucose. Phosphatidyl ethanolamine can accept methyl groups from a methyl donor, and the ethanolamine group is methylated in stages to choline so forming phosphatidyl choline. Preliminary investigations into the phospholipid content of the A. niger cells undergoing microcycle conidiation revealed a diversity of polar lipid classes with phosphatidyl choline and phosphatidyl ethanolamine as two of the major components (see Chapter 4).

The carbon skeletons from both glucose and alanine figure prominently in the formation of triacylglycerols (see Figs. 6.13 and 6.14). The triacylglycerols are a relatively prominent class at 24h when giant cell formation is complete but are less prominent when compared to fatty acid content during the latter stages of conidiation. Phosphatidic acid is an intermediate in the synthesis of traicylglycerols as well as for phospholipids.

The formation of triacylglycerols from phosphatidic acid occurs by a sequence of two reactions. Photosphatidic acid is hydrolysed in the presence of a phosphatase and the resulting diacylglycerol reacts with a fatty -acyl coenzyme A.

Fungi, in particular yeasts and Neurospora species have been extensively used in the elucidation of the pathways of sterol biosynthesis (see Weete, 1980). Squalene is an important precursor of sterols and is formed from acetyl CoA which is derived from pyruvate, the end product of glycolysis. Thus the carbon skeleton of squalene and hence sterols may be donated by glucose via glycolysis or by alanine when degraded to pyruvate. Squalene may be oxidised to epoxysqualene which can undergo cyclisation to form sterols. The first cyclic product in fungi is lanosterol which is yielded by epoxysqualene cyclase. In the fungal cell, up to 80% of the sterol content exists as sterol esters (see Weete, 1980) which are formed by the esterification of fatty acids to the 3-hydroxyl. The sterols and sterol esters derived from the carbon skeletons of both glucose and alanine make only a small contribution to the overall cellular lipid content in A. niger cells undergoing microcycle conidiation (see Figs. 6.13 and 6.14).

It is apparent from Figs. 6.13 and 6.14 that the radioisotope carbon derived from both ^{14}C glucose and ^{14}C alanine made a significant contribution to the increased fatty acid production which occurred particularly from 24 to 48h. This was a period when great energy demands were being made on the cell for the process of conidiation and the fatty acids may represent the breakdown of stored lipid reserves. Fatty acids can also be synthesised from Acetyl CoA as illustrated in Fig. 6.2.

Thus the carbon atoms for fatty acid synthesis may arise from glucose via glycolysis or from alanine by conversion to pyruvate. The pathway for the conversion of glutamic acid to TCA intermediates and hence fatty acids is not as straightforward and may explain the fact that radiolabelled carbon of glutamic acid origin was not traced to the fatty acid fraction throughout most of the 48h period of microcycle conidiation.

Further investigations are needed to assess the full impact of the carbon sources provided in the incubation medium on lipid synthesis. These could include the use of inhibitors of the enzymes involved in lipid biosynthetic pathways.

Microcycle conidiation in A. niger occurs over a forty-eight hour period during which the environmental parameters are strictly controlled. In particular, a strict temperature regime of exactly 41°C for eleven hours, followed by thirteen hours at 44°C is essential in fermenter cultivation, as is a continuous flow of a 5% carbon dioxide in air thoroughly mixed into the culture. This preliminary twenty-four hour period allows giant cell formation. A reduction in temperature to 30°C for a further twenty-four hours induces direct conidiophore production from the swollen cells, followed by completion of conidiogenesis.

The carbon source provided in the medium is mainly in the form of glucose (10.0g/l) with lesser amounts of the amino acids glutamic acid (5.0g/l in the form of the monosodium salt) and alanine (0.2g/l). The nitrogen required for growth is provided by these amino acids and an inorganic nitrogen source is supplied in the form of ammonium sulphate (2.0g/l). From the chemical analyses carried out on the medium content (Chapter 6.2.1 and 6.2.2) and the radioisotope tracer experiments (Chapter 6.2.3, 6.2.4 and 6.2.5), it is apparent that most of the glucose and alanine is taken up by the A. niger cells during microcycle conidiation. The glutamic acid is taken up to a lesser extent but with a marked increase in the rate of uptake following the reduction in temperature at 24h allowing conidiation to occur.

Under normal growth conditions at temperatures of 25-30°C, the conidia of A. niger will germinate by spherical growth reaching a maximum of 6-7 μm in diameter before germ-tube outgrowth occurs as illustrated in Fig. 3.1 (Smith and Berry, 1974).

During microcycle conidiation there is an extended period of spherical growth with the spore reaching a maximum diameter of 25 μm as reported both previously (see Smith and Berry, 1974) and in the present study. However, as discussed in Chapter 3, maximum giant cell diameter is often attained prior to 24h in the present study and conidiation is complete by 36 to 44h. This spherical swelling during microcycle conidiation is a true growth process as the decrease in medium nutrients (Figs. 6.3, 6.4 and 6.5) suggests together with the increase in cellular dry weight (Fig. 4.3), protein content (Fig. 4.4) and the incorporation of radiolabelled carbon substrates supplied in the medium into lipids and other cellular components (Figs. 6.12 and 6.9). The increase in lipid production during spore germination may be due to assimilatory growth but the increase in cellular protein content implies that autocatalytic growth occurs i.e. an increase in the nucleic acids and protein synthesis. It has been previously reported that an increase in nuclear number occurs during giant cell formation (Yahya, 1979), although levels of both DNA and RNA remained constant from 0 to 4h, but increased slowly to 16h with the greatest increase in DNA at 16 to 24h (see Smith et al., 1981).

Two phases in A. niger spore germination under normal conditions (Fig. 3.1) have been distinguished (see Smith and Berry, 1974). The first phase is swelling due to water intake and is independent of nutrients. The second phase is nutrient dependant and this indicates a period of active metabolism and growth. Heat mediated events lead to spore germination but there is controversy over the mode of spore activation.

The heat treatment may alter the protein structure of membranes or changes in the lipid phase may free membrane-bound enzymes which in turn increase the catabolic rate. Alternatively, a combination of both these processes could be involved in spore germination (Furch, 1981).

The conidia of A. niger were found to contain 0.3 µg of lipid per 1 x 10^6 cells (Fig. 4.5) which is an equivalent of 0.11% of the cellular dry weight (Fig. 4.6). Conidia of A. niger have previously been reported as containing 4.1% total lipid expressed as a percentage of cellular dry weight, of which 54.3% was polar and 45.7% neutral, although this was more saturated than was mycelial lipid (Gunasekaran et al., 1972a,c). This contrasts with the findings of the present study where 30.4% of the total lipid expressed as a percentage of dry conidial material was polar, and 69.6% neutral (Fig. 4.7). The total lipid present in conidia of Aspergillus fumigatus has been reported by Tsukahara (1980). Conidia were taken from the aerial hyphae of A. fumigatus after 4, (young conidia) and 7 days (mature conidia) growth. The total lipid present in the young conidia represented 6.0% of the total dry weight of the cells, whereas the lipid amounted to 9.9% of the cellular material in dry weight terms in the mature conidia.

The elevated temperature used in the present study to produce giant cells resulted in a content of 2.7% of cellular dry weight at 24h (Fig. 4.6). This may be compared with a total lipid content of 0.9-2.2% of the cellular dry weight of A. niger vegetative hyphae noted by Woodbine et al. (1951), and the increase during giant cell formation may be attributed to the increased temperature of incubation.

Although increased incorporation of the radiolabelled carbon from ^{14}C glucose, ^{14}C alanine and ^{14}C glutamic acid occurred from 12 to 24h, radioactivity counts at shorter intervals would be needed to pinpoint the times of greatest carbon radioisotope intake into lipids. The initiation of conidiation following the decrease in temperature to 30°C at 24h was accompanied by a marked decline in lipid content from 24 to 32h with total lipid, expressed as a percentage of cellular dry weight, decreasing from 2.6% to 0.65%. However, by 36h, when the conidiophore structure is almost complete, the lipid content had risen to 1.79% of the cellular dry weight (Fig. 4.6). The radiotracer studies show an overall increase in the incorporation of the carbon radioisotope from ^{14}C glucose, ^{14}C alanine and ^{14}C glutamic acid in the 24 to 36h period of conidiophore development. Further radiotracer analyses would be required to ascertain whether fluctuations occur in the amounts of radiolabelled carbon incorporated into the cellular lipid fraction over this twelve hour period. Similarly the decrease in the amount of total lipid present in the A. niger cells from 40 to 44h illustrated in Fig. 4.5 is not recorded on the basis of carbon radioisotope incorporation into lipids which shows an increase from 36 to 48h for all three carbon substrates. Although the amount of total lipid produced by the cells increased from 36 to 48h (Fig. 4.5), it is noteworthy that there was a marked decline in lipid expressed as a percentage of dry weight over this period (Fig. 4.6).

In contrast to fermenter cultivation, lipid accumulation by A. niger undergoing microcycle conidiation in shake-tube culture was more gradual up to 32h, increasing markedly from 32 to 40h.

The method of cultivation employed thus has an effect on lipid accumulation and it has been reported that lipid production by an oil accumulating yeast, Candida curvata D from cheese whey permeate was much more efficient by continuous culture than by batch culture (Floetenmeyer et al., 1985).

Some fermenter cultures of A. niger did not undergo the full microcycle conidiation process although environmental conditions were maintained as constant as possible from one fermenter cultivation to the next. Giant cell formation occurred without fail during the first eleven hours of the microcycle but following the increase in temperature to 44^{o}C, either autolysis or vegetative mycelial growth occurred in many cases (see Chapter 3). Kuboye et al., (1976) noted that A. niger spores formed giant cells when incubated at 41^{o}C with the same medium content as used in the present study and with the 5% carbon dioxide in air supply. They also noted that the increase in temperature to 44^{o}C at 11h prevented autolysis. It was suggested by Kuboye et al., (1976) that an exhaustion in the glucose supply resulted in autolysis and Yahya (1979) found that an increase in glucose medium content from 10g/l to 15g/l diminished the occurrence of autolysis. The autolysis of A. niger cells is accompanied by the breakdown of cell walls by lytic enzymes. A change in cell wall structure has been previously reported, accompanied by the release of substances, principally glucose and N-acetylglucosamine into the medium (Perez-Leblic et al., 1982). In the case of glucose starvation, the substances released into the medium could be used as substrates for the surviving cells.

The fact that in the present study, once autolysis set in, the procedure usually continued until each cell lysed suggests influences other than glucose starvation as the causal factors. In addition, glucose was still present at relatively high concentrations at the onset of autolysis as illustrated in Fig. 6.4).

Although an increase in glucose content of the medium in the present study resulted in spherical growth to 11h, vegetative mycelial growth occurred when the temperature was raised to 44^{o}C. Various other cultural conditions were altered in an attempt to regain the complete microcycle pattern with particular attention given to temperature control, the timing of temperature changes, growth medium content and the carbon dioxide in air supply. The A. niger conidia used to inoculate the cultures were also tested for viability and their ability to undergo the full microcycle in shake tube culture. First generation spore cultures were used where possible to reduce the risk of attenuation and a fresh culture of the A. niger strain used was obtained from the Commonwealth Mycological Institute (CMI), Kew, Surrey. Two other A. niger strains were also obtained in order to test their potential for microcycle conidiation. The overall result of all the above attempts to regain the microcycle were the persistence of autolysis and/or vegetative mycelial growth following the preliminary temperature change. The results are detailed in Chapter 3. Some confusion prevailed over the A. niger Strathclyde strain used as Aspergillus niger van Tiegham, IMI number 41873 (as used by Anderson and Smith, 1971,a,b) was ordered from the C.M.I. and was despatched as Aspergillus carbonarius.

However, an identification by the C.M.I. confirmed that the fungus used in the present work was indeed A. niger.

The fluctuations in neutral lipid levels and in particular triacylglycerol content throughout microcycle conidiation is indicative of synthesis and degradation by enzymes. Initially the A. niger giant cells and conidiating cells were tested for triacylglycerol lipase activity. Such a lipase specifically breaks down triacylglycerols releasing fatty acids. There has been much recent interest shown in the study of fungal lipases and their application in industry. The lipases from A. niger have figured prominently in recent research into enzymatic fat hydrolysis and synthesis using tallow, coconut oil and olive oil (Linfield et al., 1984). The production of lipases in A. niger grown on by-products or wastes from the food industry has been studied and the highest enzyme production occurred in batch cultures in media containing starch and sunflower seed groats (Birnescu and Vasu, 1982). A review of the properties of A. niger lipases with possible utilisations in industry has been recently prepared by Tsujisaka and Iwai (1984).

Although lipases have been isolated from many strains of A. niger e.g. A. niger isolated from groundnut (Vaidehi and Jagadamba, 1984) and A. niger NRRL 337 whose lipase synthesises an ester oligmer (Okumara et al., 1984), little detectable lipase activity was noted in the strain used in the present study of microcycle conidiation. A study of the lipolytic activity in the genus Aspergillus grown on soy flour has been investigated and of the 35 representatives tested, only 5 showed any lipase activity and even that was found to be weak and unstable. (Volkova and Lebedeva, 1979).

Further, Volkova and Lebedeva (1979) noted the necessity of a lipid component for lipase biosynthesis by Geotrichum asteroides and the stimulating influence of this component on lipase biosynthesis by Rhizopus cohnii VKM F-597. Thus the medium has a marked effect not only on the lipid content of the fungi but also on lipase production.

Esterases have been previously detected in the mycelial growth of A. niger with four types (esterase I-II-III-IV) reported by Okumara et al., (1983), two of which were markedly stable to heat. All four esterases hydrolysed aliphatic esters of short-chain fatty acids and acetyl esters of phenols but did not hydrolyse Me esters of aromatic carboxylic acids nor acetyl esters of aromatic alcohols. Lloyd et al. (1971, 1972) reported esterase presence in A. niger cells undergoing the normal asexual life cycle, but only in the later stages of conidiation. In the present study, esterase activity was detected in the conidia and throughout the period of microcycle conidiation. The esterase activity increased throughout the microcycle with the most marked increase occurring during the later stages of conidiation at 36 to 48h.

The major neutral lipid classes found in A. niger undergoing microcycle conidiation are triacylglycerols, fatty acids, sterols and sterol esters. These have all been previously reported as present in A. niger mycelial growth (Farag et al., 1979). Polar lipids are also present in A. niger cells during microcycle conidiation. They have been previously recorded as amounting to 2.2% of the total mycelial dry weight and were mainly found in the microsomal and mitochondrial fractions of the mycelia (Letoublon et al., 1982).

From the data illustrated in Fig. 4.10, it is noted that all the lipid classes are present at low levels over the preliminary stages of giant cell formation to 12h. Of these low levels, sterols, sterol esters and triacylglycerols are predominant, with fatty acids making a much smaller percentage contribution (Fig. 4.11). The final twelve hour period of giant cell formation is a period of triacylglycerol synthesis (Fig. 4.10) with an increased percentage contribution to its carbon skeleton being made by the carbon radioisotope supplied as ^{14}C glucose and ^{14}C alanine (Figs. 6.13 and 6.14). Over the 36 to 48h microcycle period, there was no net synthesis of triacylglycerol (Fig. 4.10) although there was an increased percentage incorporation of the carbon radioisotope from ^{14}C alanine accompanied by a decrease in the radiolabel from ^{14}C glucose. The increase in the fatty acid levels from 24 to 36h was accompanied by an increase in the relative percentage incorporation of the radiolabel from both ^{14}C glucose and ^{14}C alanine. During the 36 to 48h period, the fatty acid content of the A. niger cells decreased as illustrated in Fig. 4.10. Although net utilisation of fatty acid must occur over this period, the increase in the relative percentage incorporation of radiolabel from both ^{14}C glucose and ^{14}C alanine indicates that synthesis also occurred. By this stage in the microcycle, the carbon radioisotope from ^{14}C glutamic acid is also used in fatty acid synthesis (see Chapter 6.2.12).

The peak of relative percentage incorporation of radiolabel from the main carbon source, ^{14}C glucose, into polar lipids (Fig. 6.13), coincides with the peak in total polar lipid at 24h estimated by chemical analysis (Fig. 4.7).

The relative percentage incorporation of the radiolabel from both ^{14}C glucose and ^{14}C alanine decreased from 24 to 36h (Figs. 6.13 and 6.14) although there was a net synthesis of polar lipid over this period (Fig. 4.7). At this stage there was some incorporation of the carbon radioisotope from ^{14}C glutamic acid into polar lipid which could explain this increase (see Chapter 6.2.12).

It has been previously reported that 5% of available glucose was incorporated into lipid during spore germination in fungal species studied. The lipid content doubled during the first twenty-four hours of spore germination, this mainly due to saponifable (i.e. containing fatty acid) lipid. (Gottileb and Ramachandran, 1960; Van Etten and Gottileb, 1965). This data can be compared to 0.32% of the glucose taken up by the cells being used in lipid synthesis over the 0 to 12h period of the microcycle and 6.15% over the 12 to 24h period. In the present study lipid levels more than double over the first 24h of spore germination with triacylglycerols as the main lipid class synthesised.

Currently, with the advent of more sophisticated biotechnological techniques and genetic engineering, much interest is shown in the use of fungi to produce lipids and enzymes such as lipases and esterases for commercial use. Variations in cultural conditions and substrates supplied can produce an increased lipid content or a concentration of a particular lipid class in a fungus. For example, Aspergillus nidulans grown on sucrose as the carbon source contained 35.5% lipid after 21 days (Sharma et al., 1981).

The ratios and positions of saturated and unsaturated fatty acids can be altered by using specific enzymes and suitable substrates e.g. the linoleic acid specific lipase of Geotrichium candidum can be used to enrich olive oil with linoleic acid (Neidleman and Geigert, 1984).

There is also an increasing interest in running enzymatic reactions in high concentrations of organic solvents where a positive biocatalysis reaction may be achieved. Monophasic systems for ester synthesis using dried mycelia of Rhizopus arrhizus have been used with an alcohol reactant as the solvent. Also, by dissolving glycerol and fatty acid in the solvent, then contacting this mixture with dried mycelia of R. arrhizus, good yields of glycerides have been produced (Neidleman and Geigert, 1984). Such reactions could produce tailor-made glycerides for special applications.

In conclusion, it is apparent that the synthesis and utilisation of lipids, in particular triacylglycerols, fatty acids, sterols and sterol esters occurs during microcycle conidiation in A. niger. The main carbon source involved in lipid synthesis in the present study was glucose although there was a high rate of incorporation of the radiolabelled carbon supplied in the form of ^{14}C alanine throughout the cycle and also from ^{14}C glutamic acid towards the end of the microcycle. Further biochemical investigations would be required to determine the details of lipid synthesis from the substrates in the incubation medium, and to understand more fully the role of lipids in giant cell growth and conidiation in A. niger. These investigations would include assays for the enzymes of the various biosynthetic and catabolic pathways e.g. fatty acid synthetases.

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